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**Therapeutic potential of targeting group III metabotropic glutamate receptors as a disease modifying strategy in the treatment of Parkinson's disease**

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**Title:**Therapeutic potential of targeting group III metabotropic glutamate receptors as a disease modifying strategy in the treatment of Parkinson's disease

**Author:**Matt Betts

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Therapeutic potential of targeting group III  
metabotropic glutamate receptors as a disease  
modifying strategy in the treatment of  
Parkinson's disease

By

Matthew Thomas John Betts

Thesis submitted for the degree of  
DOCTOR OF PHILOSOPHY

Wolfson Centre for Age-Related Diseases  
King's College London  
September 2011

I confirm that the work presented in this thesis is my own and  
all references are cited accordingly.

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## Abstract

Parkinson's disease (PD) is characterised by a progressive loss of dopaminergic neurones from the SNpc, leading to numerous downstream changes in the basal ganglia circuitry. Overactivity of the glutamatergic subthalamonigral pathway may underlie this continual degeneration of the nigrostriatal system. With this in mind, this thesis examined whether selective activation of group III metabotropic glutamate receptor subtypes may offer a novel strategy to halt persistent degeneration in PD.

Initial distribution studies revealed mGlu4 and 7 group III mGlu receptor subtypes, demonstrated particularly intense immunoreactivity in the SNpc, suggesting these receptors may be ideally positioned to provide neuroprotective effects. Therefore, the first objective was to confirm this neuroprotective possibility using a broad spectrum agonist, L-AP4. Sub-chronic supranigral L-AP4 treatment mediated functional neuroprotection against a unilateral 6-OHDA lesion of the SN, confirmed by behavioural assessment and post-mortem analyses.

Secondly, the pharmacological identity of the group III mGlu receptor mediating this protective effect was examined. To investigate mGlu4 receptors, the novel mGlu4 selective PAM VU0155041, was also shown to provide functional neuroprotection in the 6-OHDA rat model to an almost comparable level reached with L-AP4. Whilst these neuroprotective effects are likely mediated by an inhibition of glutamate to protect from glutamate-mediated excitotoxicity, VU015504 also led to a significant reduction in levels of GFAP and IBA-1 suggesting an additional anti-inflammatory action. Further studies revealed little evidence for co-localisation of mGlu4 receptors with GFAP in the SN suggesting this anti-inflammatory component likely reflects an indirect effect via stimulation of neuronal mGlu4 receptors.

Finally, to investigate mGlu7 receptors, the selective allosteric agonist AMN082, was also shown to protect the nigrostriatal tract and demonstrate a degree of preservation of motor function. In contrast, mGlu8 receptor activation using the selective agonist DCPG, failed to protect the nigrostriatal tract or preserve motor behaviour. Collectively, these findings demonstrate that, of the group III mGlu receptors investigated, mGlu4 offers the most potential as a promising target for establishing disease modification in PD.

## Table of contents

ABSTRACT .....	2
TABLE OF CONTENTS .....	3
ACKNOWLEDGEMENTS.....	10
PUBLICATIONS RELATING TO THIS THESIS .....	11
ABBREVIATIONS.....	13

## CHAPTER 1: INTRODUCTION .....18

1.1 PARKINSON’S DISEASE.....	19
1.1.1 Prevalence, symptoms and prognosis .....	19
1.1.2 Aetiology .....	22
1.1.2.1 Genetic factors.....	22
1.1.2.2 Environmental factors .....	25
1.1.2.3 Mechanisms of nigrostriatal neurodegeneration .....	27
1.1.2.3.1 Mitochondrial dysfunction and oxidative stress .....	27
1.1.2.3.2 Glutamate-mediated excitotoxicity .....	30
1.1.2.3.3 Ubiquitin-proteasomal system .....	31
1.1.2.3.4 Neuroinflammation .....	32
1.1.2.3.5 Vulnerability of dopaminergic cells .....	33
1.2 THE BASAL GANGLIA .....	34
1.2.1 Introduction .....	34
1.2.2 Overview of the basal ganglia circuitry.....	34
1.2.3 Functional anatomy of the basal ganglia.....	37
1.2.3.1 Corticostriatal projections .....	37
1.2.3.2 Striatal interneurons .....	38
1.2.3.3 The striatofugal system .....	39
1.2.3.3.1 Direct pathway .....	39
1.2.3.3.2 Indirect pathway .....	40
1.2.3.3.2.1 External segment of the globus pallidus.....	41
1.2.3.3.2.2 The subthalamic nucleus .....	42
1.2.3.4. The thalamocortical loop .....	44
1.2.3.5. Normal basal ganglia function .....	45
1.3 THE BASAL GANGLIA IN PARKINSON’S DISEASE.....	46
1.3.1 Introduction .....	46
1.3.2 Effects of dopaminergic denervation on the striatum .....	48
1.3.3 Effects of dopaminergic denervation on the external segment of the globus pallidus and subthalamic nucleus .....	49
1.3.3.1 External segment of the globus pallidus.....	50
1.3.3.2 The subthalamic nucleus .....	51

1.3.3.2.1 Alternative routes of hyperactivity in the subthalamic nucleus .....	52
1.3.3.2.2 Nigrosubthalamic projections .....	53
1.3.3.2.3 Subthalamonigral projections to the SNpc .....	53
1.3.4 Dopaminergic denervation on the internal segment of the globus pallidus and the substantia nigra pars reticulata .....	54
1.4 ANIMAL MODELS FOR PARKINSON'S DISEASE.....	56
1.4.1 Pharmacological models.....	56
1.4.1.1 The Reserpine model.....	56
1.4.1.2 The Haloperidol model.....	58
1.4.2 Toxin-induced models.....	59
1.4.2.1 6-OHDA lesion model.....	59
1.4.2.2 MPTP-treated model.....	61
1.4.3 Pesticide-induced models.....	62
1.4.3.1 Rotenone model.....	62
1.4.3.2 Paraquat and Maneb.....	64
1.4.4 Proteasomal inhibitor models.....	64
1.4.5 Genetic models.....	65
1.5 CURRENT THERAPEUTIC STRATEGIES FOR PARKINSON'S DISEASE.....	67
1.4.1 Dopamine replacement therapy.....	67
1.4.1.1 L-DOPA .....	67
1.4.1.2 Dopamine agonists.....	70
1.4.2 Non-dopaminergic pharmacotherapy.....	71
1.4.2.1 Cholinergic drugs.....	71
1.4.2.2 Amantadine.....	72
1.4.3 Future therapeutic prospects.....	72
1.4.3.1 Neurotrophic factors.....	72
1.4.3.2 Antioxidants.....	73
1.4.3.3 Adenosine receptor antagonists .....	74
1.4.4 Non-pharmacological therapy.....	75
1.4.4.1 Stem cells and tissue transplantation.....	75
1.4.4.2 Surgical interventions.....	76
1.5 GLUTAMATERIC RECEPTORS AND THEIR POTENTIAL IN PARKINSON'S DISEASE.....	80
1.5.1 Glutamate transmission .....	80
1.5.2 Glutamate receptors .....	81
1.5.2.1 Ionotropic glutamate receptors .....	83
1.5.2.1.1 AMPA receptors: Signal transduction, general distribution and therapeutic potential in PD.....	82

1.5.2.1.2: NMDA receptors: Signal transduction, general distribution and therapeutic potential in PD.....	85
1.5.2.1.3: Kainate receptors: Signal transduction, general distribution and therapeutic potential in PD.....	87
1.5.2.2 Metabotropic glutamate receptors.....	88
1.5.2.2.1: Structure of metabotropic glutamate receptors .....	88
1.5.2.2.2 Group I mGlu receptors: Signal transduction, general distribution and therapeutic potential in PD.....	91
1.5.2.2.3 Group II mGlu receptors: Signal transduction, general distribution and therapeutic potential in PD.....	93
1.5.2.2.4 Group III mGlu receptors: Signal transduction .....	95
1.5.2.2.5 Group III mGlu receptors: General distribution .....	98
1.5.2.2.6 Group III mGlu receptors: Distribution and function in the basal ganglia .....	99
1.6 GENERAL HYPOTHESIS.....	102
1.7 BROAD AIMS OF THIS THESIS.....	103
<b>CHAPTER 2: PRELIMINARY INVESTIGATION TO ASSESS THE DISTRIBUTION OF GROUP III METABOTROPIC GLUTAMATE RECEPTORS IN THE BASAL GANGLIA OF NAÏVE RAT BRAIN .....</b>	<b>104</b>
2.1 INTRODUCTION.....	105
2.2 AIMS .....	108
2.3 METHODS .....	109
2.3.1 <i>General methodological considerations</i> .....	109
2.3.1.1 Animals .....	109
2.3.1.2 Antibodies for group III mGlu receptor subtypes .....	110
2.3.2 <i>Specific methodological details</i> .....	110
2.3.2.1 Animals .....	110
2.3.2.2 Immunohistochemical protocol .....	110
2.3.2.3 Materials and suppliers.....	112
2.4 RESULTS .....	114
2.4.1 <i>Distribution of group III mGlu receptor subtypes within the rodent basal ganglia</i> .....	114
2.4.1.1 mGlu4 receptor .....	114
2.4.1.2 mGlu7 receptor .....	116
2.4.1.3 mGlu8 receptor .....	118
2.5: DISCUSSION .....	120



# **CHAPTER 3: POTENTIAL OF TARGETING GROUP III METABOTROPIC GLUTAMATE RECEPTORS TO PROVIDE FUNCTIONAL NEUROPROTECTION IN THE 6-OHDA RAT MODEL OF PARKINSON'S DISEASE .....126**

3.1 INTRODUCTION.....	127
3.2 AIMS .....	131
3.3 METHODS .....	132
3.3.1 <i>General methodological considerations</i> .....	132
3.3.1.1 6-OHDA-lesion model of Parkinson's disease .....	132
3.3.1.2 Behavioural tests .....	134
3.3.2 <i>Specific methodological details for L-AP4 neuroprotection studies</i> .....	136
3.3.2.1 Animals .....	136
3.3.2.2 Experimental protocol for surgical cannulation.....	136
3.3.2.3 Supranigral drug administration .....	137
3.3.2.4 Intranigral 6-OHDA lesioning .....	139
3.3.2.5 Behavioural assessment.....	141
3.3.2.5.1 Cylinder Test .....	142
3.3.2.5.2 Adjusted Stepping Test .....	142
3.3.2.5.3 Amphetamine-induced rotations .....	142
3.3.2.6 Data analysis for behavioural tests.....	143
3.3.2.7 Immunohistochemistry protocol .....	145
3.3.2.8 Image and data analysis for immunohistochemical studies .....	146
3.3.2.9 Protocol for neurochemical assessment of dopamine .....	149
3.3.2.10 Data analysis for neurochemical studies .....	151
3.3.2.11 Materials and suppliers.....	151
3.3 RESULTS .....	153
3.3.1 <i>Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist, L-AP4, in unilaterally 6-OHDA-lesioned rats</i> .....	153
3.3.1.1 Effects of sub-chronic L-AP4 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta .....	153
3.3.1.2 Effects of sub-chronic L-AP4 infusion on tyrosine hydroxylase levels in the striatum .....	155
3.3.1.3 Effects of sub-chronic L-AP4 infusion on dopa decarboxylase in the striatum of 6-OHDA lesioned rats .....	158
3.3.1.4 Effects of sub-chronic L-AP4 infusion on dopamine content in the substantia nigra and striatum.....	160
3.3.1.5 Effects of sub-chronic L-AP4 infusion on motor behaviour in 6-OHDA lesioned rats.....	162
3.3.2 <i>Effects of pre-treatment with group III mGlu receptor antagonist CPPG, on L-AP4 mediated neuroprotection in unilaterally 6-OHDA-lesioned rats</i> .....	170

3.3.2.1 Effects of pre-treatment with CPPG on L-AP4 mediated neuroprotection with respect to dopamine content in the substantia nigra and striatum .....	170
4.3.2.2 Effects of pre-treatment with CPPG on L-AP4 mediated neuroprotection with respect to motor function in 6-OHDA lesioned rats .....	172
3.4 DISCUSSION .....	179
<b>CHAPTER 4: POTENTIAL OF TARGETING METABOTROPIC GLUTAMATE RECEPTOR 4 TO PROVIDE FUNCTIONAL NEUROPROTECTION IN THE 6-OHDA RAT MODEL OF PARKINSON'S DISEASE .....</b>	<b>187</b>
4.1 INTRODUCTION.....	188
4.2 AIMS .....	191
4.3 METHODS .....	192
<i>4.3.1 Specific methodological details for VU0155041 neuroprotection studies .....</i>	<i>192</i>
4.3.1.1 Animals .....	192
4.3.1.2 Surgical cannulation.....	192
4.3.1.3 Supranigral drug administration .....	192
4.3.1.4 Intranigral 6-OHDA lesioning .....	193
4.3.1.5 Behavioural assessment.....	193
4.3.1.6 Data analysis for behavioural tests.....	193
4.3.1.7 Immunohistochemical and HPLC protocols.....	194
4.3.1.8 Image and data analysis for immunohistochemistry and HPLC studies .....	196
4.3.1.9 Immunofluorescence co-localisation protocol.....	196
4.3.2.10 Materials and suppliers.....	198
4.4 RESULTS .....	199
<i>4.4.1 Initial dose finding study to investigate the effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, in unilaterally 6-OHDA-lesioned rats .....</i>	<i>199</i>
4.4.1.1 Effects of sub-chronic VU0155041 infusion on dopamine content in the substantia nigra and striatum.....	199
<i>4.4.2 Full neuroprotection study to investigate effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, in unilaterally 6-OHDA-lesioned rats .....</i>	<i>201</i>
4.4.2.1 Effects of sub-chronic VU0155041 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta .....	201
4.4.2.2 Effects of sub-chronic VU0155041 infusion on tyrosine hydroxylase levels in the striatum of 6-OHDA lesioned rats .....	203
4.4.2.3 Effects of sub-chronic VU0155041 infusion on dopa decarboxylase in the striatum of 6-OHDA lesioned rats.....	205
4.4.2.4 Effects of sub-chronic VU0155041 infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta .....	207

4.4.2.5 Effects of sub-chronic VU0155041 infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta .....	209
4.4.2.6 Effects of sub-chronic VU0155041 infusion on motor behaviour in 6-OHDA lesioned rats.....	211
4.4.3 <i>Effects of pre-treatment with group III mGlu receptor antagonist CPPG, on VU0155041 mediated neuroprotection in unilaterally 6-OHDA-lesioned rats</i> .....	218
4.4.3.1 Effects of pre-treatment with CPPG on VU0155041 mediated preservation of tyrosine hydroxylase positive cells in the substantia nigra pars compacta .....	218
4.4.3.2 Effects of pre-treatment with CPPG on VU0155041 mediated preservation of striatal dopamine content .....	220
4.4.2.3 Effects of pre-treatment with CPPG on VU0155041 mediated neuroprotection with respect to motor function in 6-OHDA lesioned rats .....	222
4.4.4 <i>Preliminary study to determine localisation of mGlu4 receptors on dopaminergic neurons and astrocytes in the SNpc of naïve rats</i> .....	228
4.4.4.1 Co-localisation of mGlu4 receptors with tyrosine hydroxylase.....	228
4.4.4.2 Co-localisation of mGlu4 receptors with glial fibrillary acidic protein .....	230
4.4 DISCUSSION .....	232
<b>CHAPTER 5: POTENTIAL OF TARGETING METABOTROPIC GLUTAMATE RECEPTOR 7 AND 8 TO PROVIDE FUNCTIONAL NEUROPROTECTION IN THE 6-OHDA RAT MODEL OF PARKINSON'S DISEASE</b> .....	238
5.1 INTRODUCTION.....	239
5.2 AIMS .....	244
5.3 METHODS .....	245
5.3.1 <i>Specific methodological details for AMN082 and DCPG neuroprotection studies</i> .....	245
5.3.1.1 Animals .....	245
5.3.1.2 Surgical cannulation.....	245
5.3.1.3 Supranigral drug administration .....	245
5.3.1.4 Intranigral 6-OHDA lesioning .....	246
5.3.1.5 Behavioural assessment.....	246
5.3.1.6 Data analysis for behavioural tests.....	246
5.3.1.7 Immunohistochemical and HPLC protocols.....	247
5.3.1.8 Image and data analysis for immunohistochemistry and HPLC studies .....	247
5.3.1.9 Materials and suppliers.....	248
5.4 RESULTS .....	249
5.4.1 <i>Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist, AMN082, in unilaterally 6-OHDA-lesioned rats</i> .....	249
5.4.1.1 Effects of sub-chronic AMN082 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta .....	249

5.4.1.2 Effects of sub-chronic AMN082 infusion on dopamine content in the striatum .....	251
5.4.1.3 Effects of sub-chronic AMN082 infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta .....	253
5.4.1.4 Effects of sub-chronic AMN082 infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta .....	255
5.4.1.5 Effects of sub-chronic AMN082 infusion on motor behaviour in 6-OHDA lesioned rats.....	257
5.4.2 <i>Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist, DCPG, in unilaterally 6-OHDA-lesioned rats</i> .....	264
5.4.2.1 Effects of sub-chronic DCPG infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta .....	264
5.4.2.2 Effects of sub-chronic DCPG infusion on dopamine content in the striatum .....	266
5.4.2.3 Effects of sub-chronic DCPG infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta .....	268
5.4.2.4 Effects of sub-chronic DCPG infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta .....	270
5.4.2.5 Effects of sub-chronic DCPG infusion on motor behaviour in 6-OHDA lesioned rats.....	272
5.4 DISCUSSION .....	279
<b>CHAPTER 6: GENERAL CONCLUSIONS</b> .....	287
REFERENCES .....	299
APPENDIX I .....	341
<i>Materials</i> .....	341
Standard solutions.....	341
Reagents and consumables.....	341
Supplier contacts .....	344

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## **Publications relating to this thesis**

### **Manuscripts**

**Betts M**, O'Neill M, Duty S (2012) Allosteric modulation of the group III mGlu receptor 4 provides functional neuroprotection in the rodent 6-OHDA model of Parkinson's disease *Br J Pharmacol* (Epub ahead of print).

Broadstock M, Austin P, **Betts M**, Duty S (2012) Antiparkinsonian potential of targeting group III metabotropic glutamate receptor subtypes in the rodent substantia nigra pars reticulata. *Br J Pharmacol. Feb;165(4b): 1034-45*

\***Betts M**, Austin P, Broadstock M, O'Neill M, Mitchell S, Duty S (2010) Symptomatic and neuroprotective effects following activation of nigral group III metabotropic glutamate receptors in rodent models of Parkinson's disease. *Br J Pharmacol. Aug;160(7):1741-53*

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### **Abstracts**

**Betts M**, O'Neill M, Mitchell S, Duty S. (2010) Selective activation of the mGlu4 but neuroprotection in the rat 6-OHDA model of Parkinson's disease. *Society for Neuroscience, San Diego, CA, USA*

**Betts M**, O'Neill M, Mitchell S, Duty S (2009) Allosteric modulation of group III metabotropic glutamate receptor 4: a potential neuroprotective approach for the treatment of Parkinson's disease. *Parkinsonism and Related Disorders 15 (Suppl 2):S121 XVIII WFN Congress on Parkinson's Disease and Related Disorders, Miami, USA*

**Betts M**, O'Neill M, Mitchell S, Duty S (2009) Supra-nigral administration of an mGlu4 receptor allosteric potentiator protects against histological and functional deficits in the 6-OHDA lesion rat model of Parkinson's disease. *British Pharmacological Society, Winter Meeting, London, UK*

**Betts M, O'Neill M, Mitchell S, Duty S (2008).** The group III mGlu receptor agonist L-AP4 affords both neurochemical and functional neuroprotection against a 6-hydroxydopamine lesion of the substantia nigra in rats. *Neuropharmacology* 55: 585-638(9) *6<sup>th</sup> International Meeting on Metabotropic Glutamate Receptors, Taormina, Sicily*

## Abbreviations

2-DG	2-deoxyglucose
3-OMD	3-O-methyldopa
5-HT	5-hydroxytryptamine/serotonin
6-OHDA	6-hydroxydopamine
ACh	Acetylcholine
AchE	Acetylcholinesterase
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
AMPA	$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
AP	Anteroposterior
ATP	Adenosine tri-phosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
BDNF	Brain derived neurotrophic factor
Bax	Bcl-2-associated X protein
CaM	Calmodulin
CDK5	Cyclin-dependent kinase 5
CK1	Casein kinase 1
CO-1	Cytochrome oxidase
COMT	Catechol-O-methyl transferase
C <sub>o</sub> Q <sub>10</sub>	Coenzyme Q <sub>10</sub> /ubiquinone
CM/ PFC	Centromedian / Parafascicular thalamic complex
CNS	Central Nervous System
CPPG	(RS)-alpha-cyclopropyl-4-phosphonophenylglycine
CR	Calretin
DAB	Diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
DARPP-32	Dopamine-and cAMP- regulated phosphoprotein
DAT	Dopamine transporter



DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DV	Dorsoventral
Dyn	Dynorphin
EAATs 1-5	Excitatory amino acid transporters 1-5
EC <sub>50</sub>	Effective concentration for 50% response
Enk	Enkephalin
EP	Entopeduncular nucleus
EPSP	Excitatory postsynaptic potential
ERK1/2	Extracellular signal-regulated kinase 1/2
ES cells	Embryonic stem cells
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GPe	External segment of the globus pallidus
GABA	$\gamma$ -aminobutyrate acid
GAD <sub>67</sub>	Glutamic acid decarboxylase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GIRK	G-protein-regulated inwardly rectifying potassium channel
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GluR1-4	AMPA receptor subunits
GPi	Internal segment of the globus pallidus
GRIP	Glutamate receptor interacting protein
GRK2	G-protein coupled receptor kinase-2
GSH	Glutathione
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBA-1	ionised calcium binding adaptor molecule 1
i.c.v.	Intracerebroventricular

iGlu	Ionotropic glutamate receptor
IL-1 $\beta$	Interleukin-1 $\beta$
i.p.	Intraperitoneal
IP <sub>3</sub>	Inositol triphosphate
IPSP	Inhibitory postsynaptic potential
KA1-2	Kainate receptor subunits
L-AAAD	L-aromatic amino acid decarboxylase
L-AP4	L(+)-2-amino-4-phosphonobutyric acid
L-DOPA	3,4-dihydroxy-L-phenylalanine
LPS	Lipopolysaccharide
LRRK2	Leucine-rich kinase 2
L-SOP	O-Phospho-L-serine
LTD	Long term depression
LTP	Long term potentiation
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MAPK	Mitogen-activated protein kinase
MFB	Median forebrain bundle
mGlu	Metabotropic glutamate receptor
ML	Mediolateral
MPP <sup>+</sup>	1-methyl-4-phenylpyridinium
MPTP	1 –methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPTP	Mitochondrial permeability transition pores
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neurone
Complex I	NADH-ubiquinone oxidoreductase (first enzyme of mitochondrial respiratory chain)
NGS	Normal goat serum
NM	Neuromelanin
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase

NPY	Neuropeptide Y
NR1-3	NMDA receptor subunits
PARK 1-13	Familial Parkinson's disease type 1-13
PBS	Phosphate buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
PFA	Paraformaldehyde
PFC	Parafascicular thalamic complex
PLC	Phospholipase C
PHCCC	N-phenyl-7-(hydroxyamino) cyclopropa[b]chromen-1a-carboxamide
PI3K	Phosphatidylinositol-3-kinase
PICK	Protein interacting with C-kinase
PINK1	Phosphatase and tensin kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PPN	Pedunculopontine nucleus
PSP	Progressive supranuclear palsy
PUMA	p53 upregulated modulator of apoptosis
PV	Parvalbumin
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RBD	REM sleep behaviour disorder
RF	Reticular formation
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
(S)-3,4-DCPG	(S)-3,4-dicarboxyphenylglycine
SC	Superior colliculus
SNCA	Gene encoding $\alpha$ -synuclein protein
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
SNP	Single nucleotide polymorphism

SP	Substance P
SS	Somatostatin
STN	Subthalamic nucleus
TBS	Tris-buffered saline
TGF- $\beta$	Transforming growth factor- $\beta$
TH	Tyrosine hydroxylase
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
TTX	Tetrodotoxin
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UPDRS	Unified Parkinson's disease rating scale
UPS	Ubiquitin-proteasomal system
VA	Vento anterior thalamic nuclei
VGLUT1-3	Vesicular glutamate transporters 1-3
VL	Ventral lateral thalamic nuclei
VM cells	Ventral mesencephalon cells
VMAT2	Vesicular monoamine transporter
VTA	Ventral tegmental area
VU0155041	( $\pm$ )-cis-2-(3,5,-dichlorophenylcarbamoyl)cyclo-hexanecarboxylic acid

## **Chapter 1: Introduction**

## **1.1 Parkinson's disease**

### **1.1.1 Prevalence, symptoms and prognosis**

Parkinson's disease (PD) was first identified by James Parkinson in 1817 in his "Essay of the shaking palsy", which describes a progressive neurological disorder consisting of resting tremor and motor disability (Parkinson, 1817), now known as idiopathic PD. Currently the second most common neurodegenerative disorder after Alzheimer's disease, PD affects 1% of the population aged 55 years or over, rising to approximately 1.5% in the 64-69 year old age group (von Campenhausen *et al.*, 2005). As these statistics indicate there is a large increase in the incidence of PD during the sixth decade of life, with the average age of diagnosis estimated to be 55 years (Bezard *et al.*, 1998). However the time of diagnosis does not reflect the age of onset of the disease since by the time PD becomes symptomatic and consequently diagnosable, it is estimated that approximately 60-70% of dopaminergic cells of the nigrostriatal tract have degenerated (German *et al.*, 1983).

PD is clinically characterised by a triad of motor symptoms namely resting tremor, rigidity and bradykinesia all of which worsen over time with the appearance of postural instability and gait dysfunction (Morris *et al.*, 2001). Additional motor symptoms frequently observed are dystonia, micrographia (decreased size of writing), hypophonia (weakened or whispered voice), drooling, inability to swallow (dysphagia) and hypomania (mask-like face with infrequent blinking) (Pallone, 2007). Aside from 'motor disturbances' numerous non-motor symptoms have also been described in PD. These include autonomic dysfunction such as constipation and urinary incontinence, psychiatric conditions such as dementia, depression, rapid eye movement (REM) sleep disorder and insomnia and in some cases hallucinations, delusions and paranoia with the latter conditions often a consequence of drug treatment (Pallone, 2007). Thus although the pathology of PD focuses largely on the degeneration of dopaminergic neurones from the nigrostriatal system, these non-motor symptoms are due to degeneration of extranigral structures. These include the noradrenergic locus coeruleus, dorsal vagal nucleus and medullary nucleus, the glutamatergic pedunculopontine nucleus (PPN), the serotonergic raphe nucleus, the

nucleus basalis of Meynert and regions of the limbic system (Jellinger, 1999). This degeneration of numerous neuronal systems implicated in PD no doubt explains the array of clinical symptoms observed with this disorder.

A major, although not exclusive, pathological hallmark of PD is the appearance of abnormal intracellular protein inclusions termed Lewy bodies, in the cytoplasm of vulnerable dopaminergic neurones of the substantia nigra pars compacta (SNpc) (Dunnett & Bjorklund, 1999). Lewy bodies, first described by Friedrich H. Lewy in 1912 are fibrous, proteinaceous inclusions containing  $\alpha$ -synuclein which are typically 15 $\mu$ m in diameter and indentified by their dense hyaline core and clear halo appearance. Lewy bodies are present in 80-100% of PD post-mortem brains (McNaught *et al.*, 2001) and according to the staging system of Lewy body pathology proposed by Braak *et al.*, (2003) are thought to precede the degeneration of dopaminergic neurones in the SNpc. Examination of synuclein pathology in post-mortem tissue from PD patients and those with no record of PD-associated symptoms, yet demonstrating Lewy body pathology revealed a definite pattern of Lewy body formation ascending from the hind brain. The brainstem, medulla and anterior olfactory nucleus are thought to be the initial sites of formation (stage 1 and 2), whilst inclusions in the SNpc which are associated with PD symptoms comprise stage 3 and 4, and finally Lewy bodies in the cortex implicated in dementia are consigned to stage 5 and 6 (Braak *et al.*, 2003). Recently, it has been suggested that two diseases associated with Lewy body pathology in stages 1 and 2 may in fact be precursors to PD. The first of which is REM sleep behaviour disorder (RBD) which refers to Lewy body pathology in the brainstem (Boeve *et al.*, 2007) and occurs in approximately 30-66% of PD sufferers (Gagnon *et al.*, 2002). It is thought more than 65% of RBD sufferers go on to develop PD and/or associated dementia (Schenck & Mahowald, 2002). Secondly, hyposmia (severe olfactory dysfunction), is thought to result from Lewy body formation in the anterior olfactory nucleus and is reported in 80-90% of PD patients (Muller *et al.*, 2002), which in some cases has been reported prior to onset of motor symptoms (Ponsen *et al.*, 2004). Indeed research in this area is very active at this moment in time in assessing the validity of both RBD and hyposmia in the prognosis of PD (Dickson *et al.*, 2009).

Despite the promising correlation between RBD and hyposmia with a pre-symptomatic phase of PD, there remain no biomarkers that can be routinely used to confirm diagnosis of PD. Therefore definitive diagnosis to date relies on post-mortem examination. Considering this along with the fact PD shares numerous clinical symptoms with dementia, Alzheimer's disease (AD) and parkinsonian syndromes such as dementia with Lewy bodies (DLB) and progressive supranuclear palsy (PSP), misdiagnosis of idiopathic PD can often occur. Indeed the poorly understood effects of co-existing pathologies on PD remain of considerable intrigue. For example, large autopsies studies have revealed that as people age they harbour numerous cellular neuropathies, suggesting manifestation of parkinsonism in the elderly may be due to a combination of pathologies within the dopamine circuitry (Litvan *et al.*, 2007). Thus the interaction between the pathologies of PD, AD and DLB requires further investigation.

The chronically progressive nature of PD can leave people almost fully immobile (or akinetic) within 10-15 years of diagnosis. Currently there remains no therapy available to halt this progressive degenerative process which results in mortality 2-5 times higher in PD patients than age-matched controls (Morens *et al.*, 1996). PD alone however is rarely identified as the primary cause of death, with pneumonia and cancer unrelated to PD, posing the highest risks of death in PD patients (Beyer *et al.*, 2001). In a time in which the number of persons over 60 globally is expected to rise by 1 billion as we reach 2050 (United Nations, 2005), the occurrence of neurodegenerative diseases is likely to reach endemic proportions. In addition to the increased suffering, the cost of care, treatment and loss of earning is likely to pose a huge economical strain in years to come. The United States economy estimated this equated to \$10.8 billion in 2009 (United Nations, 2009). Therefore it is of huge medical and economical importance to discover effective therapies to manage this disease. Ultimately, treatments which serve to halt disease progression i.e. neuroprotective agents are the most desired which ideally could be prescribed at the pre-symptomatic stage. Furthermore, it is hoped biomarkers, imaging technology and genetic screening for mutant genes ascribed to PD will be developed to identify pre-symptomatic patients for preventive treatment.



## 1.1.2 Aetiology

### 1.1.2.1 Genetic factors

Whilst it is clear genetics play a role in monogenic familial forms of PD, evidence of such a component in idiopathic PD remains controversial. Epidemiological studies and post-mortem analyses have shown 95% of patients demonstrate late onset idiopathic PD (Tanner, 2003). However, 15% of total patients with PD are thought to have a first-degree relative affected (Carr *et al.*, 2003). Nonetheless, attempts to identify autosomal dominant or recessive pro-parkinsonian genes in idiopathic cases of PD using association studies and nonparametric linkage methods have been largely unsuccessful. There remains some evidence that genetic variations of *SNCA* (the gene encoding  $\alpha$ -synuclein protein) which can cause familial monogenic parkinsonian syndromes, may alter the risk for idiopathic PD (Mueller *et al.*, 2005). The underlying concept is that relatively common genetic variations such as single nucleotide polymorphisms (SNPs), may alter the expression pattern of the gene modifying binding of transcription factors, altering splicing patterns or affecting RNA stability (Litvan *et al.*, 2007). Therefore, even though these variations do not directly result in a pathogenic form of  $\alpha$ -synuclein, small changes in protein homeostasis may shift a cell towards its threshold to neurodegeneration. In addition, it is thought, that  $\alpha$ -synuclein mutations responsible for monogenic disease can influence the risk for idiopathic PD by altering the expression level of such proteins. Thus, assessing pathogenic mutations in familial monogenic cases of PD is crucial to developing our understanding of the pathogenesis of idiopathic PD. To date, thirteen monogenic forms of PD have been identified (PARK1-13), in which the causative genes are known in six of these cases and are discussed in more detail below.

Mutations in the *SNCA* gene have been classified for causing familial type 1 Parkinson's disease (PARK1). The first reported observation of a genetic implication in PD which later became identified as alterations in PARK1, was of an Italian-American family with autosomal dominant early onset PD who were found to have a missense mutation (A53T) in the *SNCA* gene (Polymeropoulos *et al.*, 1997). Since then, numerous missense mutations in the  $\alpha$ -synuclein gene have been identified in

German (A30P) and Spanish families (E46K) which cause early onset PD by gene duplication and triplication suggesting a correlation between the number of genes implicated and disease progression (Kruger *et al.*, 1998; Zarranz *et al.*, 2004). Wild-type  $\alpha$ -synuclein has been shown to accumulate in the presynaptic nerve terminals implicated in dopaminergic transmission and vesicle dynamics (Abeliovich *et al.*, 2000; Outeiro & Lindquist, 2003). However cellular localisation of studies of mutant versions of  $\alpha$ -synuclein have shown deposition in Lewy bodies and Lewy neurites (Spillantini *et al.*, 1997). The ubiquitin-proteasome system (UPS) disposes of abnormal proteins through a complex series of reactions preventing toxic accumulation. However, the increased expression of these mutant  $\alpha$ -synuclein aggregates is believed to impair their ubiquitination leading to toxic accumulation (discussed in more detail in section 1.1.2.3.3).

The discovery of Parkin (PARK2) originates from Japanese families found to have autosomal recessive juvenile parkinsonism (Kitada *et al.*, 1998). Exonic deletions in the gene encoding the Parkin protein was discovered in approximately 50% of familial early-onset patients in which 77% of cases were in juvenile patients (onset < 20 years) yet only in 3% of cases with late onset (>30 years, Lucking *et al.*, 2000). For this reason and owing to the limited Lewy body pathology in this familial form of PD it is thought the mutated Parkin gene has little implication in idiopathic PD. However some SNPs have been associated with increased expression of wild-type Parkin. Indeed non mutated Parkin has been found in the protein aggregate of Lewy bodies from idiopathic PD patients (Schlossmacher *et al.*, 2002). It is now thought that Parkin and  $\alpha$ -synuclein may interact via an intermediate protein synphylin-1 (Chung *et al.*, 2001) which may be implicated in the pathological formation of Lewy bodies.

Mutations in the UCHL1 gene (PARK5) resulting in a heterozygous I93H substitution, have also been identified from a pair of siblings affected by PD (Leroy *et al.*, 1998). Ubiquitin carboxyl-terminal esterase L1 (UCHL1) along with a de-ubiquitinating enzyme, and the ubiquitin protein ligase Parkin comprise components of the UPS which collectively serve to degrade abnormal protein. It would be pertinent to suggest therefore that mutations in UCHL1 and Parkin prevent the

effective disposal of abnormal proteins, leading to toxic accumulation and possible cell death (discussed in more detail in section 1.1.2.3.3).

Additional mutations have been discovered in the gene encoding phosphatase and tensin kinase 1 (PINK1), which have been shown to cause familial type 6 PD (PARK6) (Valente *et al.*, 2004). PINK1 mutations comprise approximately 1-7% of familial cases (Healy *et al.*, 2004; Rohe *et al.*, 2004) and monogenic forms of early-onset PD resemble those of a form of idiopathic PD with atypical features, such as dystonia and psychiatric complications (Healy *et al.*, 2004; Tan *et al.*, 2006). Transfection of mutant PINK1 protein in HEK293 cells has been shown to prevent atractyloside-induced opening of mitochondrial permeability transition pores (mPTP) permitting the release of apoptogenic cytochrome c from the mitochondria (Wang *et al.*, 2007a). Interestingly the same study revealed over-expression of wild-type PINK1 mediated neuroprotection through inhibition of pro-apoptotic, Bcl-2-associated X protein (Bax) and prevented opening of mPTP. The authors go on to suggest the apoptotic mechanism of mPTP opening may be due to a Ser/Thr kinase mutation to PINK and inability to downregulate Bax. These findings would suggest PINK1 gene mutations may be implicated in mitochondrial dysfunction (discussed in greater detail in section 1.1.2.3.1), which is thought to be implicated in the pathology of PD.

Mutations in the DJ-1 gene (PARK7) were first reported to cause a monogenic form of early onset PD in two European families (Bonifati *et al.*, 2003). It is thought however many mutations in DJ-1 do not cause familial PD and account for less than 1% of all cases of familial PD. In the few cases in which DJ-1 is implicated, neurodegeneration from mutant forms is believed to be mediated via oxidative stress (discussed in greater detail in section 1.1.2.3.1). Although thought to comprise a minor role in familial cases it is interesting that in idiopathic PD, higher plasma levels of DJ-1 were found in patients with early onset PD compared to age-matched controls. Furthermore DJ-1 levels were higher still in patients with advanced disease progression (Waragai *et al.*, 2007). These findings taken together would suggest levels of DJ-1 may correlate to disease progression and consequently may serve as a marker to detect pre-symptomatic PD.

Finally, mutations in leucine-rich kinase 2 (LRRK2) have also been shown to cause monogenic forms of PD (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004). Through extensive screening in a range of ethnic populations the most common LRRK2 protein mutation (G2019S), has been found to be responsible for 5-19% of idiopathic cases (Goldwurm *et al.*, 2005; Hernandez *et al.*, 2005a; Litvan *et al.*, 2007; Zimprich *et al.*, 2004). Generally, subjects indicative of this form of familial PD demonstrate idiopathic pathology i.e. presence of Lewy bodies and degeneration of dopaminergic neurones of the nigrostriatal system (Hernandez *et al.*, 2005b). However, it currently remains unclear how mutations of the LRRK2 protein directly cause PD. Since some mutations in LRRK2 have been shown to cause late-onset PD indistinguishable from idiopathic disease (which is a unique characteristic amongst the PARK genes), further unravelling of the role of LRRK2 protein is now underway with the hope of providing valuable insight into the pathogenesis of idiopathic PD.

#### **1.1.2.2 Environmental factors**

The previous section clearly delineates a strong genetic contribution to the initiation of early-onset PD, although other factors are also likely to be involved. This has been best demonstrated in a study in which an octogenarian carrier of the LRRK2 G2019S mutation has been shown to be neurologically healthy (Kew & Kemp, 2005). This observation suggests genetic factors may simply predispose carriers of mutant genes to other risk factors. This is an appealing suggestion considering a lack of strong evidence for a genetic contribution in idiopathic cases, proposing environmental and also epigenetic factors may be of equal importance in disease progression.

In 1979, it was discovered that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant in a batch of heroin, induced an irreversible juvenile-onset parkinsonian syndrome in Californian drug addicts (Langston *et al.*, 1983). It is now widely documented that MPTP when it is converted to its active metabolite 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) in astrocytes by monoamine oxidase and then taken up into dopaminergic neurons via the dopamine transporter (DAT), causes a state of oxidative stress (Javitch *et al.*, 1984). Toxicity of MPP<sup>+</sup> lies in its ability to inhibit the first enzyme of the respiratory chain, NADH-ubiquinone oxidoreductase (complex I)

generating reactive oxygen species and consequent mitochondrial dysfunction leading to dopaminergic cell death, discussed in more detail in section 1.1.2.3.1 (Nicklas *et al.*, 1987; Sherer *et al.*, 2002). Owing to its specificity to induce dopaminergic degeneration, MPTP has become one of the most widely used toxins to model PD in mice and primates.

A common household pesticide, rotenone, has also been shown to cause parkinsonism in rats where these animals demonstrate Lewy-body like inclusions containing  $\alpha$ -synuclein (Betarbet *et al.*, 1997). Like MPTP, rotenone is known to act as an inhibitor to complex I (Ramsay *et al.*, 1991), leading to the suggestion it may also induce PD in humans through mitochondrial dysfunction. Additionally, an agricultural herbicide paraquat, has also been shown to be toxic to dopaminergic cells, supported by a study demonstrating nigrostriatal degeneration following chronic exposure in mice (McCormack *et al.*, 2002). Furthermore, chronic exposure to paraquat in mice has been shown to cause upregulation of  $\alpha$ -synuclein in the substantia nigra (SN; Manning-Bog *et al.*, 2002), posing a link between paraquat induced nigral damage and genetic processes. Finally, paraquat is also implicated in inhibiting the mitochondrial electron-transport demonstrating non-selective inhibition at all respiratory chain complexes (Nis-Oliveira *et al.*, 2006).

Other potential environmental risk factors for PD include heavy metal exposure, which has been supported by numerous epidemiological studies. Copper, iron, aluminium, manganese and zinc are all associated with an increased incidence of PD in metal workers with exposure of 20 years or longer (Gorell *et al.*, 1997). The exact neurotoxic mechanism exerted by metals is unclear, however in the case of iron, zinc and copper, it is thought these metals initiate the production of free radicals to facilitate a state of oxidative stress (Montgomery, 1995).

Aside from factors implicated in the pathogenesis of idiopathic PD, both smoking and caffeine have been shown to negatively correlate with the incidence of PD. Indeed, Ross *et al.*, (2000) demonstrated in one study the risk of PD in non-coffee drinkers was more than five times that compared to coffee drinkers. Caffeine is a known antagonist at adenosine A<sub>2A</sub> receptors which are largely restricted to the striatum

(Fink *et al.*, 1992), hence blockade of these receptors may underlie neuroprotection mediated by caffeine. In support of this notion, systemic administration of the A2A antagonist 8-(3-chlorostyryl) caffeine has been shown to attenuate a decrease in nigral tyrosine hydroxylase (TH) immunoreactivity induced by 6-OHDA confirming a neuroprotective role for these receptors (Bove *et al.*, 2005). Furthermore, epidemiological studies have confirmed that there is an inverse correlation between smoking and PD, in which these effects are dose-dependent (Gorell *et al.*, 1999). Additionally experimental findings have shown nicotine elevates dopamine transmission in the striatum and further provides neuroprotection in an animal model of PD (O'Neill *et al.*, 2002; Visanji *et al.*, 2006).

Taken together, these findings present strong evidence that environmental toxins pose an important risk to PD, whilst in some cases may serve to alleviate this tendency. Therefore the general consensus within the literature accepts environmental factors are not exclusively responsible for idiopathic PD, which likely involves a combination including genetic and epigenetic factors.

### **1.1.2.3 Mechanisms of nigrostriatal neurodegeneration**

Despite an extensive research effort spanning greater than 50 years since the first discovery that marked striatal dopamine deficiency is responsible for PD (Ehringer & Hornykiewicz, 1960) the underlying cause of nigral cell degeneration remains elusive, although several mechanisms have been proposed.

#### *1.1.2.3.1 Mitochondrial dysfunction and oxidative stress*

Considerable evidence supports a major role for mitochondrial dysfunction and oxidative stress in the pathogenesis of PD. Under normal conditions tight regulation of the production and removal of reactive oxygen species (ROS) enables effective mitochondrial metabolism for the production of ATP. ATP is produced by the transport of protons across the inner mitochondrial membrane via the use of an electron-transport chain in which the final destination for an electron along the chain is an oxygen molecule. In approximately 1-2% of cases, oxygen is reduced which can

lead to the formation of superoxide radicals. These radicals are highly unstable, therefore can easily react with mitochondrial DNA to alter the mitochondrial membrane potential to facilitate damaging processes. Under normal conditions, antioxidants such as Vitamin C, glutathione (GSH) and thiodeoxin serve to scavenge these radicals to maintain a below toxic threshold. However, mitochondrial dysfunction as a result of inhibition to complex I of the respiratory chain, disrupts the flow of electrons along the electron –transport chain, leading to the increased production of superoxide radicals to levels that cannot be maintained by antioxidant defence systems. Furthermore superoxide radicals can react with nitric oxide to produce the extremely reactive peroxynitrite (Packer *et al.*, 1996). Accumulation of both these oxidative and nitrative stressors ultimately leads to cellular damage, precipitating both necrotic and apoptotic cell death (Tan *et al.*, 1998).

Mitochondrial complex I-mediated oxidative stress is implicated in PD patients, with an approximate 30% reduction in complex I activity seen in the SNpc of PD patients post-mortem (Schapira *et al.*, 1989). As previously mentioned, MPTP, rotenone and paraquat also inhibit complex I and are strongly implicated in inducing a PD-like syndrome in animals and in the case of MPTP, primates and humans. Dopaminergic cell death by MPTP supports a function for oxidative and nitrative stressors since both inhibition of neuronal nitric oxide synthase (which produces nitric oxide) and increased activity of superoxide dismutase (breaks down superoxide radicals) prevent MPTP induced parkinsonism in animal models (Ferrante *et al.*, 1999; Przedborski *et al.*, 1995). Free radical production by the Fenton reaction leading to  $\text{Fe}^{2+}$ -induced oxidative stress (Olanow & Arendash, 1994) may also be implicated in PD since specific increases in  $\text{Fe}^{2+}$  have been found in the SNpc of PD patients post-mortem (Dexter *et al.*, 1989).

Additional production of ROS is driven through altered dopamine metabolism. As a result of uncoupling of the electron-transport chain, inhibition of mitochondrial complex I reduces production of ATP. Therefore processes dependent on ATP such as packaging of dopamine into cellular vesicles via the vesicular monoamine transporter (VMAT) become disrupted leading to a rise in cytosolic dopamine. Owing to the extremely reactive nature of oxidative free radicals, the elevated levels of

cytosolic dopamine can react to form radical intermediates such as quinones and semiquinones (Graham, 1978) leading to oxidative stress. Through their interaction with quinones, dopamine in this instance can also reduce the levels of the antioxidant GSH, further contributing to oxidative damage by reducing available GSH to detoxify such oxidative stressors. Indeed, in the laboratory, GSH depletion has been shown to induce a strong and transient intracellular increase in ROS (Merad-Boudia *et al.*, 1998). Furthermore, this depletion in GSH was linked to altered activity in complexes I, II and IV of the electron transport chain. Numerous studies have since compounded the importance of GSH in the pathology of PD whereby many separate reports demonstrate decreased levels of GSH in the SNpc of PD patients (reviewed by Mayer, 2005). It is widely accepted GSH serves an essential role in maintaining the integrity of mitochondrial function to prevent a state of oxidative stress which can be severely challenged by oxidative and nitrative stressors.

Mitochondrial dysfunction and oxidative stress has also been implicated in genetic and familial PD. Giasson *et al.*, (2000) have shown extensive nitrated tyrosine residues of  $\alpha$ -synuclein within Lewy bodies and neurites suggesting there may be a correlation between the extent of oxidative and nitrative damage with the pathogenesis of PD (Giasson *et al.*, 2000). Prior to its association with PD, DJ-1 was thought to function as an inducible antioxidant on exposure to oxidative stimuli (Mitsumoto & Nakagawa, 2001). Furthermore an experimental study has revealed inhibition of DJ-1 leads to the accumulation of ROS, enhanced vulnerability to oxidative stress and ultimately degeneration of dopaminergic neurones (Yang *et al.*, 2005). Oxidative damage of DJ-1 has also been identified in the brains of PD patients with idiopathic PD (Choi *et al.*, 2006).

Overall there is a wealth of evidence that mitochondrial dysfunction and oxidative stress comprise a significant role in both idiopathic and familial forms of PD. This is likely through a combination of both an elevation in production of ROS and additional free radicals, in addition to a reduction in antioxidants such as GSH. One notion is that the pathogenesis of PD may be explained by the fact neurones of the SNpc are preferentially damaged by inhibitors of complex I as shown by studies using MPTP and rotenone *in vivo* (Betarbet *et al.*, 2000; McCrodden *et al.*, 1990).



This would imply dopaminergic neurones of the SNpc may be particularly vulnerable to higher levels of oxidative stress compared to other brain regions.

#### *1.1.2.3.2 Glutamate-mediated excitotoxicity*

It was first appreciated that excitatory amino acids could be neurotoxic in 1957 when Lucas & Newhouse found that high levels of glutamate given systemically to mice could induce damage of retinal neurones (Lucas & Newhouse, 1957). It is now known that following glutamate activation of ionotropic  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors there is subsequent  $\text{Na}^+$  entry and neuronal depolarisation. However if the cell becomes chronically depolarised through continuous glutamate activation, the  $\text{Mg}^{2+}$  blockade of N-methyl-D-aspartate (NMDA) receptors is released enabling their activation by glutamate causing a huge influx of  $\text{Ca}^{2+}$  (Doble, 1999). This triggers a cascade of intracellular events, driven by activation of intracellular  $\text{Ca}^{2+}$ -dependent enzymes, which disrupt the cell homeostasis leading to cell death. These enzymes include nucleases which disrupt the organisation of chromatin, causing DNA fragmentation (McConkey *et al.*, 1988); cytosolic proteases, such as calpain, which attack the cytoskeleton (Siman *et al.*, 1989); kinases such as protein kinase C which modify the phosphorylation state of cytoplasmic proteins disrupting cell function (Favaron *et al.*, 1990); and finally lipases such as phospholipase  $\text{A}_2$ , which attack the cell membrane and further organelles (Doble, 1999). Furthermore, high extracellular glutamate can inhibit the cells antioxidant defence mechanisms by inhibiting the uptake of cysteine required for GSH production resulting in elevated levels of ROS (McNaught *et al.*, 2006).

A number of observations (Dawson & Dawson, 1998; Greene & Greenamyre, 1996) have demonstrated a potential source of excess glutamate in PD is the hyperactive subthalamic nucleus (STN) which has glutamatergic projections to the SNpc and may well induce glutamate mediated excitotoxicity of dopaminergic neurones. This theory forms the rationale for targeting the over-activity of the STN to protect from glutamate-mediated excitotoxicity and fundamentally underpins the main hypothesis examined in this thesis. The aforementioned impairment to the mitochondria may also lead to 'indirect' excitotoxicity since normal maintenance of the membrane

potential by ATP is lost, resulting in membrane depolarisation and expulsion of voltage-gated  $Mg^{2+}$  block. As a result, normal (non-toxic) levels of glutamate are able to activate NMDA receptors leading to  $Ca^{2+}$  influx and consequent excitotoxicity (Greenamyre, 2001).

#### *1.1.2.3.3 Ubiquitin-proteasomal system*

Within the last decade, the ubiquitin-proteasomal system (UPS) has also been implicated in the pathogenesis of PD largely attributed to the discovery of mutations in UPS members, Parkin, and UCHL1 in familial PD (previously discussed in section 1.1.2.1). The UPS is a system essential in the removal and degradation of non-lysosomal mutant, misfolded or mislocated proteins in eukaryotic cells (McNaught *et al.*, 2001). The pathway comprises several enzyme-dependent reactions to enable selective targeting of ubiquitin-protein conjugates which can then be identified and consequently degraded by 26/20S proteasomes (primary enzymes involved in protein degradation within cells). The products of degradation can then be recycled to produce new proteins.

In PD however, proteolytic activity of the 26/20S proteasomes are reduced by up to 45% in the SN due to structural and functional defects in the 26/20S proteasome (McNaught *et al.*, 2001). This prevents the complete degradation of misfolded, mutant and mislocated proteins leading to protein aggregation, including the formation of Lewy bodies. Currently, it remains a topic of controversy whether this process is a contributor to disease progression or a cytoprotective response to other toxic events (McNaught *et al.*, 2006). In support of a pathogenic role, treatment with proteasomal inhibitors such as lactacystin, or epoxomicin led to selective degeneration of dopaminergic cells in culture (McNaught *et al.*, 2002b; Miwa *et al.*, 2005; Rideout *et al.*, 2005; Rideout *et al.*, 2001) and degeneration of the nigrostriatal system accompanied with motor dysfunction in rats (Fornai *et al.*, 2003; McNaught *et al.*, 2002a). In addition, neuronal degeneration in these studies was accompanied with the accumulation of  $\alpha$ -synuclein and ubiquitin containing Lewy body-like inclusions. In PD, UPS function can be further influenced by inhibition of Complex I of the mitochondria inhibiting ATP synthesis since ubiquitination, de-ubiquitination and

degradation via the 26/20S proteasome are ATP-dependent processes (McNaught *et al.*, 2001). This no doubt contributes to the observed accumulation of cytotoxic ubiquitin containing proteins and  $\alpha$ -synuclein on application of a complex I inhibitor rotenone. Therefore although the exact role of the UPS in PD remains unclear, there are numerous findings which suggest alterations in normal function contribute to the pathogenesis of PD.

#### *1.1.2.3.4 Neuroinflammation*

Considerable evidence suggests activation of neuroinflammatory cells provokes the process of neurodegeneration. Microglia operate as the resident immunocompetent and phagocytic cells in the CNS, serving a critical role in maintaining normal function. However it is thought chronic activation of microglia may cause neuronal damage through the release of cytotoxic molecules such as pro-inflammatory cytokines, ROS and proteinases (Dheen *et al.*, 2007). An elevation in pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$ , tumour necrosis factor (TNF- $\alpha$ ), and interferon- $\gamma$  have been found in the striatum, SNpc and cerebrospinal fluid of PD patients (Boka *et al.*, 1994; Hunot *et al.*, 1999; Nagatsu *et al.*, 2000) suggesting the normal activity of microglia is compromised. Indeed, reactive microglia has been observed in the substantia nigra of patients up to 16 years following exposure to MPTP (Langston *et al.*, 1999). Furthermore, the increase in microglial pro-inflammatory cytokines are specific to the area of injury, an example of this is the cytokine IFN- $\gamma$  which has been located exclusively in the SN of PD patients (Boka *et al.*, 1994). It is thought the cytotoxic effects of pro-inflammatory cytokines relate to the activation of inducible nitric oxide synthase (iNOS) generating high levels of nitric oxide. This in turn creates nitrosylated proteins such as nitro-tyrosine, for example from tyrosine hydroxylase (TH), leading to oxidative stress. This has been supported by an observed increase in 3-nitrotyrosine immunostaining in Lewy bodies of PD patients (Good *et al.*, 1998).

#### 1.1.2.3.5 Vulnerability of dopaminergic cells

Considering the previously described mechanisms, dopaminergic cells are thought to be particularly vulnerable to neurodegeneration and extensive efforts have been made to elucidate the impact of this contributing factor to the pathogenesis of PD. On closer inspection, degeneration of the SNpc in PD is not uniform as there appears to be a subset of selectively vulnerable neurones in the ventrolateral sensorimotor tier of the SNpc. Cell loss in this region has been shown to correlate well with levels of DAT expression (Sanghera *et al.*, 1997), suggesting increased intracellular dopamine levels may lead to the accumulation of harmful dopamine oxidation products, or alternatively an increased transport of environmental toxins such as rotenone or MPTP (discussed in section 1.1.2.2). In fact, five subsets of vulnerable nigral cells within the SNpc, termed nigrosomes have been described (Damier *et al.*, 1999) which all demonstrate low levels of the cytoplasmic protein calbindin. Under normal conditions, calbindin regulates intracellular  $\text{Ca}^{2+}$  to a level not deemed toxic by the surrounding cells (as discussed in section 1.1.2.3.2). Therefore a reduction in calbindin may well expose vulnerable cells to neurodegeneration by excess  $\text{Ca}^{2+}$ . Furthermore the vulnerability of dopaminergic neurones in the SNpc has been linked to their high levels of neuromelanin (NM) pigment. In human tissue from PD patients, increased expression of NM has been shown in degenerate dopaminergic neurones of the SNpc, whereby neighbouring healthy dopaminergic cells demonstrate no increase in NM (Halliday *et al.*, 2005). While the function of NM is not entirely clear, it has a complex structure associating with proteins and lipids, leading to oxidative stress and iron loading (discussed in section 1.1.2.3.1), as well as possible precipitation of  $\alpha$ -synuclein around NM lipids. Therefore, it is thought that increases in NM may prelude neurodegeneration in vulnerable neurones of the SNpc.

To summarise, it is unlikely there remains a single causative factor for the initiation of idiopathic PD rather a complex interaction of genetic, epigenetic and environmental factors is involved. These may include genetic mutations which may lead to the altered expression of proteins such as  $\alpha$ -synuclein involved in the pathogenesis of PD, in addition to exposure of environmental toxins such as pesticides and heavy metals. The ensuing mechanisms of nigrostriatal degeneration

are equally complex, likely implicating numerous mechanisms such as mitochondrial dysfunction, oxidative stress, excitotoxicity, UPS dysfunction and neuroinflammation. Although there is a wealth of evidence to confirm their involvement, the precise order in which these mechanisms are implicated in the neurodegenerative process remains to be determined.

## **1.2 The basal ganglia**

### **1.2.1 Introduction**

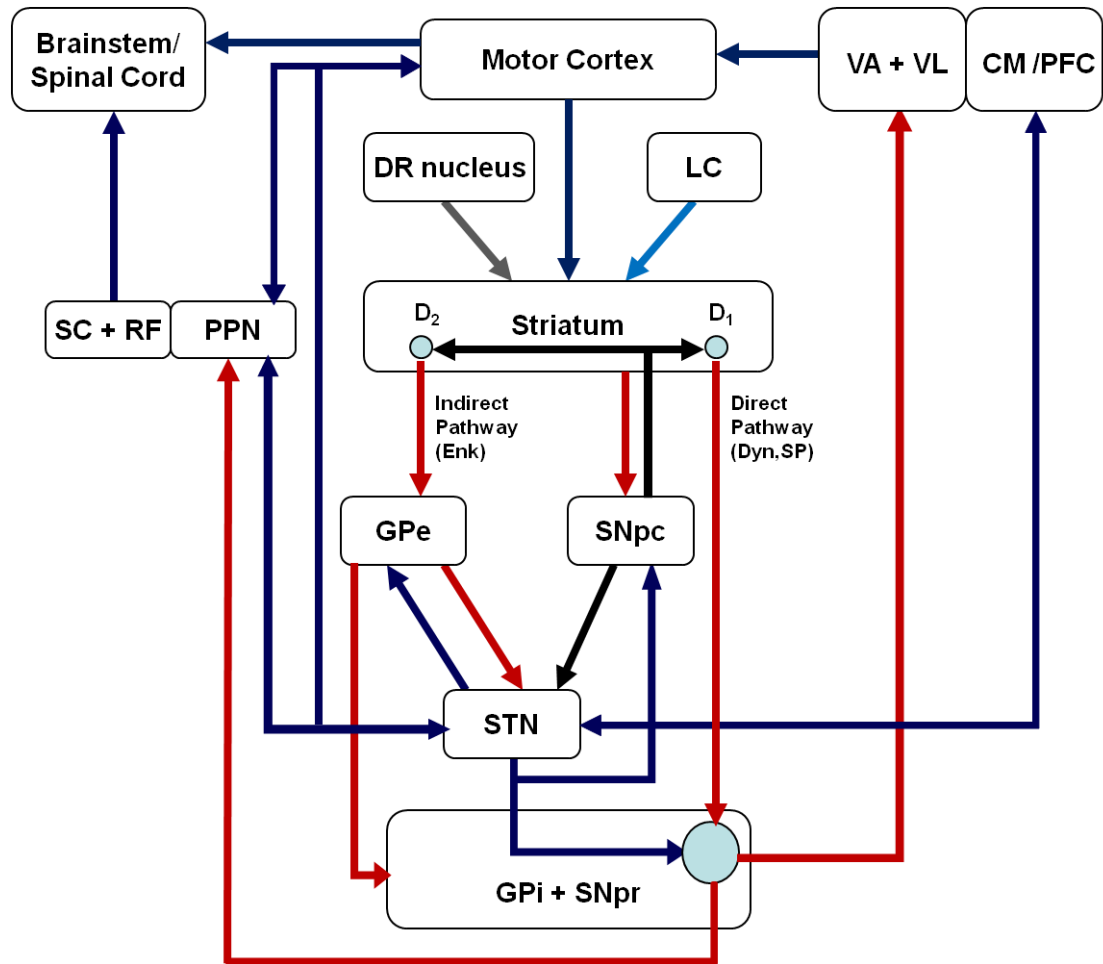
In order to appreciate the pathophysiology outlined in PD it is important to consider the functional organisation of the basal ganglia (also referred to as the ‘extrapyramidal’ system) in maintaining normal motor function. Five different basal ganglia-thalamocortical circuits have been identified, involved in controlling motor function, cognition and emotion (Alexander *et al.*, 1986). Since PD is predominantly a movement disorder, the most relevant to this work is the basal ganglia-thalamocortical circuitry implicated in regulating motor function.

### **1.2.2 Overview of the basal ganglia circuitry**

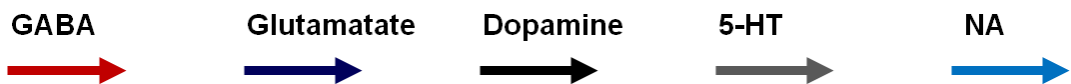
Located in the basal telencephalon, the basal ganglia (BG) consists of five interconnecting nuclei; comprising the caudate nucleus, putamen, and globus pallidus which is anatomically divided into an external component (GPe) and internal component (GPi or the Endopenducular nucleus (EP) in the rodent) segments. Furthermore the substantia nigra, separated into the dopaminergic substantia nigra pars compacta (SNpc) and the GABAergic pars reticulata (SNpr) together with the subthalamic nucleus (STN) complete the motor loop. In primates a partial separation between the caudate nucleus and putamen by the internal capsule exists, however due to the overlap in both anatomical and functional characteristics the structures will collectively be referred to here as the striatum. Indeed in rodents, the species of focus in these studies, the striatum exists as a homogenous structure (Blandini *et al.*, 2000). A simplified schematic of the organisation of the basal ganglia and other connected nuclei are illustrated in figure 1.1. It is important to note that basal ganglia nuclei do

not form direct connections with spinal motor neurones, therefore the role of the basal ganglia motor loop is not to provide initiation of movement. Rather, it is thought the basal ganglia serves to refine movement via intermediary control of strength of muscle contractions, and organisation of the requisite sequences of excitation of cortical cell columns. The functional components of the basal ganglia comprise the input regions, mainly the striatum (although the STN receives some cortical input) and output structures such as the SNpr and the GPi, juxtaposed between the STN, GPe and SNc. An anatomical layout of this kind enables the basal ganglia motor loop to integrate and modulate cortical input to be relayed to the thalamus and ultimately back to the cortex.

Figure 1.1: Interconnecting circuitry of the basal ganglia and associated nuclei under normal conditions.



**Principal Neurotransmitter:**



**Figure 1.1.** Simplified schematic of the basal ganglia motor circuitry and connecting nuclei under normal physiological conditions. VA/ VL: Venteroanterior/ ventrolateral thalamic nuclei; CM/PFC: Centromedian/ Parafascicular thalamic complex; GPi: internal globus pallidus; SNpr: Substantia nigra pars reticulata; STN: Subthalamic nucleus; GPe: external globus pallidus; SNpc: Substantia nigra pars compacta; D<sub>1</sub>, D<sub>2</sub>: Dopamine receptor subtypes; LC: Locus coeruleus; DR nucleus: Dorsal raphé nucleus; PPN: Pedunculopontine Nucleus; SC: Superior colliculus; RF: Reticular formation, 5-HT: 5-hydroxytryptamine (serotonin); NA: Noradrenaline.

### **1.2.3 Functional anatomy of the basal ganglia**

#### **1.2.3.1 Corticostriatal projections**

The initial corticostriatal projections serve as the starting point for the flow of motor information through the basal ganglia. Cortical input to the striatum occurs from all areas of the cortex, although with differing degrees of intensity; for example, the visual cortex has much fewer projections than the sensorimotor cortex, which makes extensive striatal connections (Berendse *et al.*, 1992). Since the cortex is organised in a highly topographical manner, it follows that the corticostriatal projection can be viewed as the transposition of the functional cortical map onto the basal ganglia; and accordingly the striatum is topographically arranged into distinct functional domains, such as associative, sensorimotor and limbic modalities (reviewed in Parent & Hazrati, 1995). These different areas receive input from associative (frontal, temporal and parietal lobes), sensorimotor (primary motor cortices and sensorimotor cortex) and limbic (limbic cortices, amygdala and hippocampus) areas respectively (Parent & Hazrati, 1995). This organisation into functional domains, with is believed to allow integration of cortical information passing through the striatum. The targets of the corticostriatal projections are both major striatal compartments, the striosomes (or patches) and the extrastriosomal matrix, where these projections form asymmetric (excitatory glutamatergic) synapses on striatal medium spiny neurons (Smith & Bolam, 1990). However, this idea is not supported by the observation that individual striatal neurons do not share more than approximately 1% of cortical afferents, implying each striatal neuron receives a unique cortical input (Kincaid *et al.*, 1998). The cortical input to the striatum is further integrated with glutamatergic inputs from the midline and intralaminar nuclei of the thalamus, serotonergic input from the dorsal raphè nucleus, noradrenergic input from the locus coeruleus, and crucially to this work, dopaminergic input from the SNpc (discussed in greater detail in section 1.2.3.6).



### 1.2.3.2 Striatal interneurons

Striatal interneurons are morphologically characterised by the absence of spines on their dendrites and account for 1 in 9 striatal neurones in the rodent and 1 in 3 in primates (Graveland & Difiglia, 1985). These interneurons can be further subdivided into two main categories; cholinergic giant aspiny interneurons and GABAergic medium-sized interneurons. The giant aspiny interneurons account for between 1 and 2% of the striatal neuronal population, and have a large (20-50µm diameter) cell body, with extensive axonal collaterals. Being cholinergic in nature, these giant aspiny neurons represent the major source of acetylcholine within the striatum. They are predominantly innervated by glutamatergic neurons from the corticostriatal and thalamocortical pathway. They also receive a minor dopaminergic input from the nigrostriatal pathway, with dopamine being able to modulate acetylcholine release by actions at the D2-type dopamine receptor (Chang, 1988). Cholinergic synapses have also been shown to synapse symmetrically onto medium spiny neuron dendrites and spines, suggesting a role for ACh in the modulation of striatal output.

Medium-sized GABAergic interneurons can again be subdivided into three main categories, based on their neurochemistry and physiology. These are GABA, co-expressed with either parvalbumin (PV), calretinin (CR) or neuropeptide Y (NPY), somatostatin (SS) and NADPH diaphorase (Kawaguchi *et al.*, 1995). Important species differences exist in interneuron subtype expression with PV-positive striatal interneurons being the most common subtype in rats, accounting for around 1% of the total, whereas CR expressing interneurons are the most abundant in the striatum of human and nonhuman primates (Levesque *et al.*, 2003). PV-containing interneurons have been the most widely characterised which make multiple synaptic connections with medium spiny neurons. These multiple connections permit potent feed-forward inhibition of spiny neurons, with single interneurons being able to inhibit spike occurrences (Tepper *et al.*, 2004). Furthermore it has also been shown that 99% of GABAergic medium aspiny interneurons also express tyrosine hydroxylase (TH) suggesting co-expression with dopamine (Betarbet *et al.*, 1997). Experimentally it has been shown that striatal TH increases following a nigral lesion which may act as a possible compensatory mechanism to counteract the loss of dopamine in the SN

(Huot *et al.*, 2008; Tande *et al.*, 2006). Dopamine production by these interneurons has also been shown to stimulate growth factors (Bedard *et al.*, 2006), posing an intriguing possibility that interneurons could replace nigral dopamine lost in PD.

### **1.2.3.3 The striatofugal system**

A clear majority (95%) of output neurones from the striatum are the GABAergic medium spiny neurones (MSNs), collectively referred to as the striatofugal system. In this system information is relayed by MSNs from the striatum to three further basal ganglia nuclei, namely the GPi, GPe and the SNpr. The functional segregation of inputs to the striatum (associative, sensorimotor and limbic) is maintained, leading to topographical maps within striatal output structures too. Whilst MSN projections have traditionally been divided into those projecting to the GPi, GPe or the SNpr, anatomical studies in the rodent have suggested this may be an oversimplification. This is evidenced by MSNs giving rise to extensive local axon collaterals such that the three pathways are synaptically connected (Yung *et al.*, 1996). Additionally, in monkeys up to 90% of striatofugal fibres arborise in all three target structures (Levesque *et al.*, 2003), although each axon appears to have a preferential target structure, where upon it arborises extensively (Parent *et al.*, 2001). Accepting this limitation, the striatofugal projections have been divided into two distinct populations termed the direct and indirect pathways based on their target structure, function and neurotransmitter co-localisation.

#### *1.2.3.3.1 Direct pathway*

The direct pathway projects directly from the striatum straight to the output nuclei of the basal ganglia, the GPi and the SNpr. The so-called striatonigral projections of the direct pathway are comprised predominantly of D<sub>1</sub> receptor expressing medium spiny neurones which co-utilise the predominant neurotransmitter GABA, with either dynorphin and / or substance P (Beckstead & Kersey, 1985; Smith *et al.*, 1998). Although initially thought to be a single entity, the two output nuclei are now thought to exert independent roles. The SNpr receives greater input from the associative striatal modality with some evidence of cross-talk between the three striatal

modalities. In the GPi however, the three striatal modalities remain separate where the predominant input is from the sensorimotor striatal modality (Parent & Hazrati, 1995). This topographical organisation of the striatonigral projection supports the view that cortical input to the striatum is then functionally subdivided into differing modalities, which when processed return to the cortical area from which they originated. The striatopallidal projections are also thought to be topographically organised in this way. For example a study in rodents using retrograde tracers has revealed the dorsal striatum projects to the dorsal EP whilst the ventral striatum projects to the ventral EP (Fink-Jensen & Mikkelsen, 1989). However it is thought there is huge convergence of information leaving the striatum owing to the small cell numbers in the output nuclei in comparison to the striatal projection fibres. For example, a rodent study identified 27 million striatal neurones in comparison to 26,300 cells in the SNpr and 3,200 cells in the EP suggesting considerable integration of striatal modalities.

Activation of the normally quiescent inhibitory direct pathway results in inhibition of the output nuclei of the basal ganglia. Consequently this leads to disinhibition of the GABAergic projection neurones of the SNpr/GPi releasing the motor thalamus from tonic inhibition. As a result, this causes activation of thalamocortical glutamatergic neurones in the motor cortex leading to an overall increase in movement.

#### *1.2.3.3.2 Indirect pathway*

In contrast to the direct pathway, striatal information is transferred via two additional brain nuclei, namely the GPe and STN before passing to the output nuclei. Also in contrast to the direct pathway, these MSNs express the inhibitory,  $G_{i/o}$  coupled  $D_2$  receptor subtypes at their cell bodies, have GABA and enkephalin as their co-transmitters and project almost exclusively to the GPe (Beckstead & Kersey, 1985). Thus, striatal GABAergic projection neurones connect firstly to the GPe before passing either mono-synaptically to the SNpr / GPi, or to the subthalamic nucleus (STN). The STN is a glutamatergic nuclei and information directed via this route comprises the classical indirect pathway (Smith *et al.*, 1998). Activation of the first part of the indirect pathway termed the striatopallidal pathway results in inhibition of GPe neurones which serves to alleviate the tonic inhibition of STN neurones leading

to an increase in excitatory drive to the basal ganglia output structures. This enhanced activation of the inhibitory output nuclei further inhibits thalamic feedback to the motor cortex, resulting in decreased movement. Thus activation of the indirect pathways modulates movement with opposing physiological effects to the direct pathway.

#### *1.2.3.3.2.1 External segment of the globus pallidus*

The GPe receives two main inputs: a GABAergic pathway from the striatum, and a glutamatergic pathway from the STN. In addition, less prominent inputs have been observed from the SNpc, the dorsal raphe nucleus and the pedunculopontine nucleus. As with the nigrostriatal pathway, the topographical organisation of the striatum is maintained within the GPe. For example, the sensorimotor modality is maintained along striatopallidal projections forming rostrocaudal bands within the ventrolateral GPe (Parent, 1990). The main pallidofugal targets are the STN, the SNpr / GPi and to a lesser extent, the striatum. Classically, the GPe was viewed as no more than a relay to the STN, permitting the excitatory glutamatergic inputs from the STN to counter-balance the inhibitory GABAergic striatal inputs within the SNpr / GPi. However, this view has been challenged with the discovery of the pallidostriatal and pallidonigral pathways, suggesting the GPe exerts more of a modulatory role with greater complexity in the GPe output neurones than originally thought. Indeed on the basis of their axonal targets, GABAergic GPe neurons can be divided into four main groups: (i) neurons projecting to the GPi, STN and SNpr (ii) neurons projecting to the GPi and STN (iii) neurons targeting the STN and SNpr and (iv) neurons projecting to the striatum (Parent *et al.*, 2001). Owing to the numerous target projections presented to the GPe, this would suggest considerable plasticity may exist within the basal ganglia nuclei however this is a poorly understood notion at this time. Thus for simplicity, GPe projection neurones from this point will be considered as having one primary target nuclei only.

The pallidostriatal pathway projects to the PV-positive GABAergic interneurons, forming contacts on their proximal regions. Thus, the pallidostriatal pathway can reciprocally control striatofugal pathway activity, via selective inhibition of these

interneurons which project to MSNs (Bolam *et al.*, 2000). The alternative ‘indirect pathway’ projects to the SNpr / GPi, and forms symmetrical synapses with the proximal dendrites of these neurons. This arrangement may allow pallidal modulation of either striatal or subthalamic afferents within the output nuclei, permitting a further level of modulation of basal ganglia output.

The pallidosubthalamic projection comprises the majority of the remaining GPe efferent neurons forming the principal inhibitory projection to the STN (Fonnum *et al.*, 1978). Through these inhibitory connections, the GPe is able to exert powerful modulatory actions upon the STN, by directly suppressing spontaneous firing of STN neurons or indirectly inhibiting the excitation of STN neurons through additional afferents such as corticosubthalamic afferents (Parent & Hazrati, 1995). The GPe also receives a powerful reciprocal excitatory input from the STN to form the GPe-STN loop, which is believed to form a shared control mechanism, whereby activation of one nucleus is able to control the activity of the other. The importance of this GPe-STN loop is evident from the pathophysiological changes in the activity of this pathway in PD, discussed below.

#### *1.2.3.3.2.2 The subthalamic nucleus*

The STN is the only glutamatergic nucleus of the basal ganglia, which is also topographically organised, with the dorso-caudal third of the nucleus relating to the sensorimotor modality (Joel & Weiner, 1997). The STN receives inhibitory afferents from the aforementioned pallidosubthalamic pathway as well as from the ventral pallidum and ventral striatum (Groenewegen *et al.*, 1993; Kita, 1993). Opposing excitatory innervations of the STN is received from numerous sources such as the corticosubthalamic (glutamatergic), thalamosubthalamic (glutamatergic), nigrosubthalamic (dopaminergic), the cholinergic and glutamatergic pedunculopontine-subthalamic (cholinergic and glutamatergic), raphe-subthalamic (serotonergic) projections and parafascicular-subthalamic (glutamatergic) projections (Canteras *et al.*, 1990; Fujimoto & Kita, 1993; Hedreen, 1999; Lavoie & Parent, 1990).

The direct corticosubthalamic pathway arises predominantly from the primary and supplementary motor areas in the cortex which project to the dorsal aspects of the STN (Nambu *et al.*, 1996). Corticosubthalamic projections are mainly composed of axonal collaterals from the pyramidal tract, however some cortical fibres jointly innervate the striatum (Hamani *et al.*, 2004). These axons make asymmetric glutamatergic synapses with the small dendrites of subthalamic neurons which predominantly project to the GPe. Electrical activation of the primary motor cortex thereby results in two excitatory peaks separated by a brief period of inhibition within the STN. The origin of the initial excitatory response is the direct mono-synaptic corticosubthalamic pathway. After the initial excitatory response from the STN, the inhibitory response then arises through reciprocal interactions between the STN and GABAergic efferents of the GPe. The second excitatory response is complex and less well understood involving a loop between the nucleus accumbens and ventral pallidum to drive disinhibition of the STN (Maurice *et al.*, 1998).

Comprising the main efferent projections of the STN are the subthalamopallidal and subthalamonigral pathways although smaller projections to the striatum, PPN and parafascicular thalamic complex (PFC) have also been described (Parent & Hazrati, 1995). Except for exclusive connections to the GPe and striatum (10.7% and 17.3% of total projections respectively), subthalamic projection neurones have been shown to innervate numerous targets in primates. These can be divided into three main groups based on their target nucleus beginning with those that project to the GPi and GPe (48%), those that project to the SNpr, GPi and GPe (21.3%) and finally those that project to the GPe and SNpr (2.7%) (Sato *et al.*, 2000). Thus the main target of the STN in 82.7% of cases is the GPe in which these subthalamopallidal projections form asymmetric glutamatergic synapses on dendritic shafts which lie in dense bands. The organisation of these bands is similar to that observed with the striatopallidal pathway, and ultrastructural studies have shown convergence of inputs from both striatopallidal and subthalamopallidal pathways onto the same pallidal output neurons. Hence, activity of the GPe is likely controlled by counterbalancing this excitatory activity of the subthalamopallidal projections with the inhibitory striatopallidal projections. This subthalamopallidal projection back to the GPe completes the reciprocal loop between the GPe and the STN. The other

subthalamopallidal (subthalamo-entopeduncular nucleus) pathway is formed from collaterals of STN efferents innervating the GPi, although this is formed predominantly of collaterals which innervate the GPe, thus forming one half of the classical indirect pathway.

As mentioned, the subthalamonigral projection is of primary focus in the studies described in this thesis, therefore its key role in the indirect pathway is discussed below. Subthalamonigral fibres target both segments of the SN, although the majority of these fibres selectively innervate the SNpr. The remainder innervate the SNpc, forming part of an STN-SNpc feedback loop (discussed in more detail in section 1.3.3.2.3). This loop comprises excitatory STN efferents to the SNpc which drives dopamine release (Groenewegen *et al.*, 1993); whilst SNpc projections to the STN are believed to activate inhibitory D<sub>2</sub> receptors (Hassani *et al.*, 1997) (discussed in section 1.3.3.2.2). Upon entry to the SN, subthalamonigral neurons arborise extensively, forming local collaterals which predominantly contact dendritic shafts of nigral neurons (Hamani *et al.*, 2004). Whilst this pathway accounts for the majority of glutamatergic neurotransmission within the SN, overall glutamatergic synapses only account for a minority (approximately 10%) of the total synapses present, with GABAergic symmetrical synapses accounting for the majority (Rinvik & Ottersen, 1993). With STN projections to the GPi, the subthalamonigral pathway completes the classical indirect pathway. Activation of these excitatory subthalamonigral projections drives nigral inhibition of thalamocortical loops, resulting in an overall net decrease in movement. Furthermore the subthalamonigral projection neurons have also been shown to converge with striatonigral and pallidonigral inputs, upon single nigral target neurons (Kolomiets *et al.*, 2003). This convergence of inputs onto single nigral cells permits interaction between the direct pathway, with both projections of the indirect pathway to modulate basal ganglia output.

#### **1.2.3.4. The thalamocortical loop**

The thalamus facilitates the passage of information from the basal ganglia output nuclei (SNpr/GPi) back to the cortex. Consistent with the basal ganglia nuclei described, there are several topographically organised thalamocortical loops

innervating distinct cortical areas. Traditionally, the motor thalamus refers to the more ventral thalamic region which is usually sub-divided into two major territories; the ventral anterior thalamic nuclei (VA) which receives input largely from the basal ganglia, and the ventral lateral thalamic nuclei (VL) which receives afferents predominantly from the cerebellum. The glutamatergic VA pathways predominantly innervate caudal cortical regions and are primarily involved in motor function, whilst those projections from the VL nuclei innervate the rostral cortical areas and are primarily involved in cognitive aspects of motor function, including motor learning (Haber & McFarland, 2001). Whilst the exact mechanisms by which the thalamocortical loop serves to modify motor behaviour are not yet known, this final link within the basal ganglia is well placed to integrate limbic, associative and sensorimotor inputs to impact motor behaviour.

#### **1.2.3.5. Normal basal ganglia function**

Under resting conditions the overall net output of the basal ganglia is thought to be one of inhibition. This arises from the tonically active inhibitory GABAergic projections from the SNpr and GPi to the thalamic nuclei and further to the cortex in addition to brainstem pre-motor regions such as the PPN, the superior colliculus (SC) and the reticular formation (RF) (Parent & Hazrati, 1995). Resting conditions are maintained by balancing the activity of both the direct and the indirect pathways of the striatofugal system. Thus activation of the direct pathway relieves tonic inhibition of thalamocortical feedback whereas activation of either indirect pathway acts to maintain this inhibition. In order to initiate movement, activation of the direct corticostriato-pallidal projections results in inhibition of the output nuclei, and resultant disinhibition of thalamic nuclei, which in turn leads to activation of the thalamocortical feedback loop, returning to the motor cortex. Conversely, execution of movement arises from activation of the indirect corticostriatal-pallidal-subthalamic circuit. This leads to stimulation of the output nuclei, resulting in inhibition of thalamo-cortical afferents. The temporal balance between these two pathways is such that following direct pathway activation, and resulting initiation of movement, indirect pathway activation serves to either focus the resultant movement, or inhibit further unwanted movements (Haber & McFarland, 2001). This complex interplay of



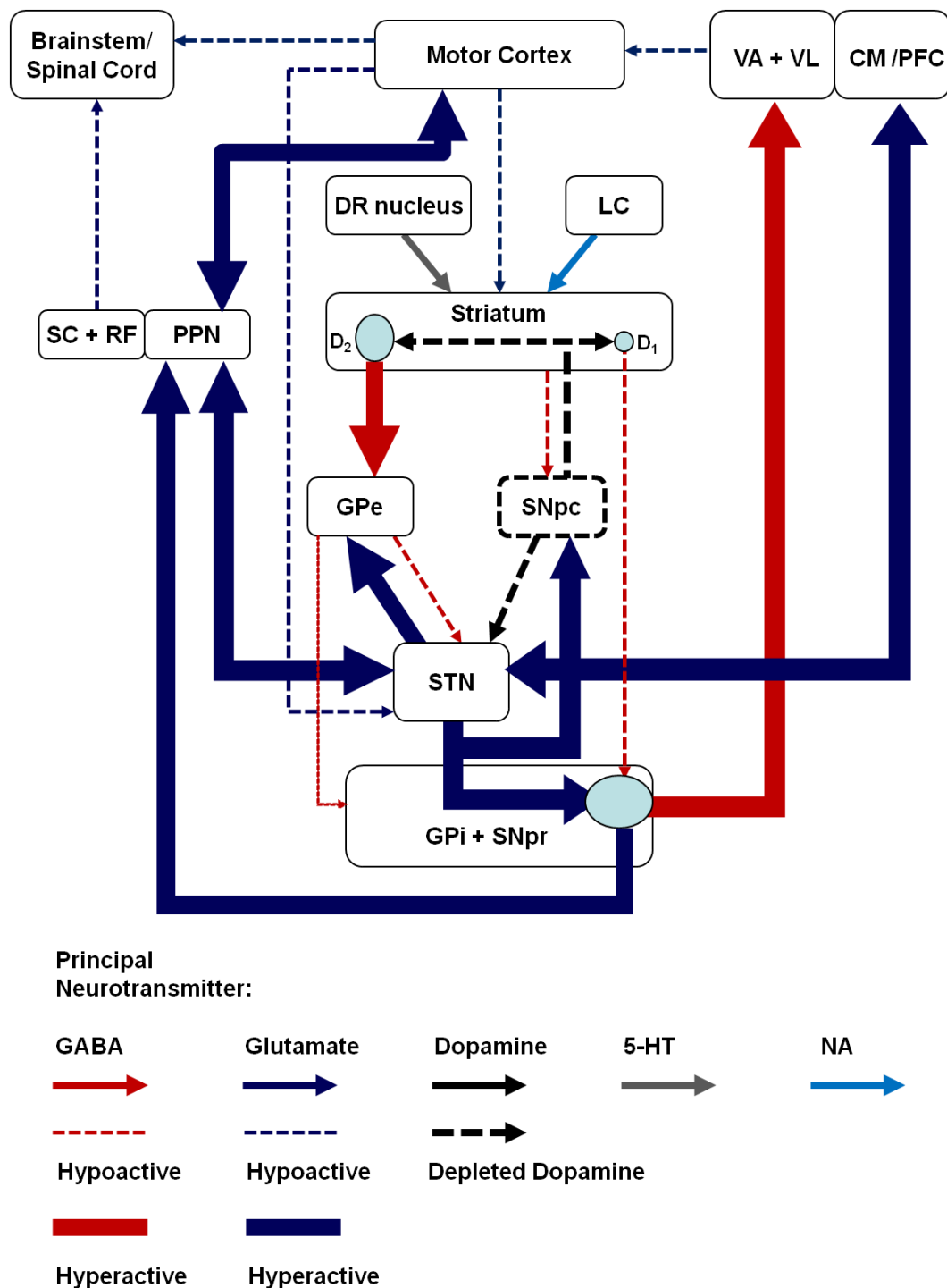
pathway activation is dependent on integration of the actions of numerous neurotransmitters, loss of which results in basal ganglia dysfunction and resultant aberrations in motor control, as will now be discussed in PD.

### **1.3 The basal ganglia in Parkinson's disease**

#### **1.3.1 Introduction**

As previously discussed in section 1.1.1 of this chapter, it is the dopaminergic denervation of the striatum that is thought to underlie the motor symptoms associated with PD. Nonetheless onset of degeneration of the nigrostriatal pathway precedes the onset of symptoms by some years. This is in part due to the compensatory mechanisms taken into effect by remaining nigral and striatal dopaminergic neurones to counteract the loss of dopamine (Zigmond *et al.*, 1990). However a point is reached in which the ongoing degenerative process reaches a severity whereby remaining intact dopaminergic neurones are no longer able to compensate the ensuing degeneration. This leads initially to decreased stimulation of both pre- and postsynaptic dopamine receptors within the striatum followed by numerous alterations in the basal ganglia circuitry such as modulation of corticostriatal input and disruptions in the striatofugal system. These alterations in the basal ganglia circuitry are summarised in figure 1.2.

Figure 1.2: Alterations within the interconnecting circuitry of the basal ganglia and associated nuclei in Parkinson's disease.



**Figure 1.2.** Simplified schematic of the basal ganglia motor circuitry in Parkinson's disease. Abbreviations: VA/VL: Ventroanterior/ ventrolateral thalamic nuclei; CM/PFC: Centromedian/ Parafascicular thalamic complex; GPi: internal globus pallidus; SNpc/r: Substantia nigra pars compacta/reticulata; STN: Subthalamic nucleus; GPe: external globus pallidus; D<sub>1</sub>, D<sub>2</sub>: Dopamine receptor subtypes; LC: Locus coeruleus; DR nucleus: Dorsal raphe nucleus; PPN: Pedunculo pontine Nucleus; SC: Superior colliculus; RF: Reticular formation; 5-HT: 5-hydroxytryptamine (serotonin); NA: Noradrenaline.

### 1.3.2 Effects of dopaminergic denervation on the striatum

Under normal conditions, pre-synaptic D<sub>2</sub> receptors on glutamatergic corticostriatal afferents serve to regulate glutamate release. However under parkinsonian conditions, loss of striatal dopamine leads to an increase in glutamatergic stimulation of the striatum. This loss of control of glutamatergic transmission results in increased AMPA and NMDA mediated excitation, due to loss of dopamine mediated corticostriatal long term depression (LTD) and consequent increased basal activity in MSNs (Calabresi *et al.*, 1996).

Furthermore, loss of dopamine from the striatum also results in changes in activity of both the direct and indirect pathways as a result of reduced stimulation of postsynaptic dopamine D<sub>1</sub> and D<sub>2</sub> receptors. Owing to their opposing influences, this leads to two main effects. Firstly the direct pathway becomes hypoactive as D<sub>1</sub> receptor mediated excitation on the striatonigral pathway is lost. Secondly the indirect pathway becomes hyperactive owing to the loss of D<sub>2</sub> receptor mediated inhibition. Consequently this leads to inhibition of the GPe and further disinhibition of the STN, to a level in which the STN becomes hyperactive. This hyperactivity within the excitatory subthalamonigral pathway, coupled with the loss of the inhibitory striatonigral pathway leads to a net increase in activity of the GPi and SNpr. As a result this increased activity of the output nuclei inhibits thalamic relay nuclei consequently inhibiting thalamocortical feedback. It is the inhibition of thalamocortical feedback that is thought to underlie the akinetic and bradykinetic symptoms of PD.

In addition to the influences on the activity of the GABAergic striatofugal pathways described above, dopamine depletion also leads to alterations in the expression of peptides and receptors within these pathways. In the direct pathway, the loss of excitatory effects of dopamine mediated by the D<sub>1</sub> receptors results in decreased expression of both Substance P and dynorphin. Conversely, the loss of D<sub>2</sub>-mediated inhibition in the indirect pathway has been linked with an increase in enkephalin expression. It has also been suggested that alterations in peptide expression form an endogenous compensatory mechanism to counteract the loss of dopamine, which has

been supported by electrophysiological studies that have shown enkephalin can decrease inhibitory post-synaptic potentials (IPSPs) in GP neurones, presumed by activation of delta opioid receptors (Stanford & Cooper, 1999). These findings suggest that levels of expression of co-transmitter correlate well with activity within the direct and indirect pathway in which peptides are able to modulate activity directly.

### **1.3.3 Effects of dopaminergic denervation on the external segment of the globus pallidus and subthalamic nucleus**

The alterations in activity within the striatofugal pathways proceed to influence downstream changes in the other basal ganglia structures including the GPe and STN. Crucially to the experimental chapters described in this thesis are the implications attributed to hyperactivity of the STN, therefore this particular aspect of the parkinsonian basal ganglia will be discussed in greater detail than the effects on the GPe.

Ever since the model of basal ganglia anatomy and function was proposed by Albin and colleagues in 1989, which suggested for the first time an explanation of how dopamine deficiency led to motor disturbances in PD, the activity of the GPe and STN in the parkinsonian basal ganglia has been contentious. According to this classical model, loss of the dopaminergic nigrostriatal pathway results in reduced activity of the GPe which in turn disinhibits the STN, resulting in STN hyperactivity. This view was supported by initial experimental evidence assessing metabolic activity in both the GPe and STN by means of 2-deoxyglucose (2-DG), (a marker of synaptic afferent activity). In addition, ablation of the nigrostriatal tract by MPTP increased 2-DG uptake in the GPe (Crossman *et al.*, 1985; Mitchell *et al.*, 1989), but decreased uptake in the STN (Mitchell *et al.*, 1989), suggesting hyperactivity in the striatopallidal tract, but decreased activity in the pallidosubthalamic pathway, as proposed by the model of Albin. Owing to the reciprocal anatomical connections with the STN, this would suggest hypoactivity in the GPe may well be implemented in the hyperactivity of the STN.

### 1.3.3.1 External segment of the globus pallidus

The classical model of the basal ganglia circuitry has come under scrutiny over the debate surrounding the hypoactive state of the GPe in PD. Previous studies using 2-DG to assess the activity of the GPe and STN have been questioned as changes on 2-DG may also reflect alterations in intrinsic neurone activity rather than changes in GABAergic inputs. Additionally, 2-DG uptake does not differentiate between excitatory or inhibitory inputs to either nucleus, nor does it distinguish between presynaptic terminal activity or postsynaptic transmission (Parent & Cicchetti, 1998). With this in mind, *in situ* hybridisation studies of glutamic acid decarboxylase (GAD<sub>67</sub> – enzyme responsible for the synthesis of GABA) are considered to be a far more accurate measure of GABAergic activity. Using this method, it has been shown that there is in fact no difference in GABAergic activity in the GPe between MPTP primates or PD patients and their controls (Levy *et al.*, 1997). In this report, metabolic activity in the GPe neurones was also examined by assessing the activity in the first subunit of cytochrome oxidase (CO-1), an established marker of neuronal functional activity. In support of findings from the *in situ* studies, CO-1 activity was not altered in MPTP-treated primates or PD patients on comparison to their respective controls (Levy *et al.*, 1997).

On the contrary, decreased firing rates by up to 20% in the GPe of MPTP-treated monkeys and 6-OHDA-lesion rats have been demonstrated suggesting a hypoactive GPe (Filion & Tremblay, 1991; Pan & Walters, 1988). However in both these studies, these effects were associated with alterations in firing pattern to a burst-type firing. This actually may result in no net decrease in GPe activity at all, as burst-type firing is more suggestive of an excitatory stimulation when compared to normal. It is more likely that the GPe does not fire at a decreased rate in PD, rather that the loss of dopamine modifies the pattern of firing in GPe neurones. Moreover, lesioning of the pallidsubthalamic pathway induces only a slight increase (20%) in STN discharge rates and no change in the firing pattern when compared to control rats (Hassani *et al.*, 1996). However, in 6-OHDA-lesioned rats a greater increase in STN firing was observed (106%), with a burst-type firing pattern (Hassani *et al.*, 1996). These findings crucially demonstrate the increased firing of the STN neurones following

dopamine depletion are not solely due to a hypoactive GPe. This suggests there are likely additional, alternate pathways that may drive STN hyperactivity.

### **1.3.3.2 The subthalamic nucleus**

Hyperactivity of the STN has been well documented both in animal models of PD and in PD patients. For example, electrical measurements of the spontaneous firing rate of STN neurons in MPTP-treated primates was shown to increase to 26Hz in comparison to 19Hz recorded in control animals, equating to a 27% increase in STN firing rates (Bergman *et al.*, 1994). Additionally, biochemical dopamine depletion in reserpine and  $\alpha$ -methyl-para-tyrosine-treated rats, led to a 53% increase in firing rates of STN neurones (Robledo & Feger, 1991). Furthermore, following chronic striatal dopamine depletion by 6-OHDA, STN neurons demonstrated discharge rates exceeding 233.3% of non-lesioned animals (Magill *et al.*, 2001). In human PD patients, two microelectrode studies measuring firing in STN neurones have shown firing rates of 37 Hz and 41Hz comparable to those seen in primate studies with MPTP (Hutchison *et al.*, 1998; Magnin *et al.*, 2000). As in animal models of PD, high-frequency burst-type STN firing was also seen in 47% of STN cells recorded in PD patients with resting tremor whilst no such activity was observed in patients without resting tremor (Levy *et al.*, 2000). These findings would suggest the high frequency burst-type firing observed here may be associated with the rhythmic pathology underlying tremor in PD patients.

A number of downstream changes in basal ganglia circuitry are supportive of hyperactivity of the glutamatergic STN. The first line of evidence equates to the downregulation of glutamate receptors in the projection nuclei of the STN (namely the SNpr, GPi and GPe), observed in 6-OHDA lesioned rodents (Porter *et al.*, 1994; Wullner *et al.*, 1994) presumably in response to enhanced glutamatergic stimulation from the STN. Indeed in PD patients, downregulation of NMDA receptors in the GPi has been observed in support of these animal studies (Lange *et al.*, 1997). In addition a microdialysis study in 6-OHDA-lesioned rats has shown 10 to 16 fold increases in glutamate levels in the SNpr and GP respectively, compared to non-lesioned animals 3 weeks post-lesion (Windels *et al.*, 2005).

Additional evidence for a hyperactive STN, and subsequent increase in glutamatergic transmission in the GPi / SNpr contributes to the motor symptoms of PD is supported by the antiparkinsonian efficacy of subthalamotomy, which serves to reduce excessive glutamatergic transmission to the SNpr (Alvarez *et al.*, 2005) Furthermore, systemic administration of the NMDA and AMPA antagonists, MK-801 and LY293558 respectively, has been shown to demonstrate antiparkinsonian efficacy in 6-OHDA-lesioned rats (Vila *et al.*, 1999). Indeed, targeting of hyperactive glutamatergic subthalamic terminals is the rationale behind various therapeutic approaches, discussed in sections 1.4.3 and 1.5 and comprise the focus of studies performed in this thesis.

#### *1.3.3.2.1 Alternative routes of hyperactivity in the subthalamic nucleus*

If a decrease in inhibitory transmission from the GPe does not solely underlie the increase in STN activity, it is possible that a number of other excitatory inputs could be involved. As mentioned, excitatory glutamatergic inputs to the STN arise from the PPN, the PFC and the cerebral cortex. *In situ* hybridisation for CO-1 mRNA in STN afferent neurones, from the PPN and PFC, revealed a 123% and 63% increase respectively in 6-OHDA-lesioned rodents compared to sham animals (Orieux *et al.*, 2000). This same study also revealed there was no significant effect on CO-1 activity in the GPe. These findings together suggest that the increase in glutamatergic transmission from the PFC and the PPN but not the GPe may underlie some of the elevated STN activity. However with respect to the PPN, closer examination of the literature would suggest the role of this nucleus in driving subthalamic hyperactivity in PD may be somewhat controversial, with evidence in favour of a hyperactive but also hypoactive role.

If indeed alterations in PPN activity are responsible for the hyperactivity in the STN, one suggestion that may forge contradictions in the literature together, is that hyperactivity in GABAergic terminals from the GPi and SNpr drives IPSP generation in the PPN, leading to strong rebound spike generation in the PPN (Kang & Kitai, 1990). It may be this rebound activity of the PPN that could drive STN hyperactivity,

therefore the PPN activity state may change during disease progression. In support of this hypothesis, the GABA<sub>A</sub>-receptor antagonist, bicuculline, when directly injected into the PPN is able to alleviate akinesia in the MPTP-treated monkey to a magnitude comparable to those seen with oral L-DOPA (Nandi *et al.*, 2002). Therefore the initial consideration of whether the PPN is hypo or hyperactive is likely far too simplistic where there is clearly a more complex mechanism involved here. A similar situation is presented on assessing the activity of the PFC in which evidence in favour of increased and decreased glutamatergic innervations from the STN have been reported (Henderson *et al.*, 2000; Orioux *et al.*, 2000). Whilst it is clear glutamatergic excitatory afferent connections from the PPN, PFC and cortex are all potential candidates to increase activity in the STN in the parkinsonian basal ganglia, their contribution to this cause remains uncertain.

#### *1.3.3.2.2 Nigrosubthalamic projections*

One further possibility for the hyperactivity of the STN is that the degeneration of the SNpc initiates changes in dopaminergic transmission in extra-striatal regions. Although nigrosubthalamic projections are certainly in the minority compared to nigrostriatal projections, degeneration of this pathway may result in similar changes to those observed in the striatum. As mentioned previously, in normal conditions dopamine released from nigrosubthalamic projections act on D<sub>2</sub> receptors in the STN, to maintain an inhibitory hold on glutamatergic subthalamonigral activity (Hassani *et al.*, 1997). In PD, degeneration of the nigrosubthalamic pathway has been demonstrated (Francois *et al.*, 2000) which would be expected to increase the activity of the STN through loss of D<sub>2</sub> receptor mediated inhibition, however this hypothesis has not been proven experimentally.

#### *1.3.3.2.3 Subthalamonigral projections to the SNpc*

Reciprocal subthalamonigral projections to the SNpc have been identified as having a key pathological role in the neurodegeneration of the nigrostriatal pathway. As discussed in some detail, the activity of the STN has a profound impact on the parkinsonian basal ganglia. Thus, increased glutamate release from the



subthalamonigral terminals onto dopaminergic cells of the SNpc may cause glutamate mediated excitotoxicity resulting in cell death within the SNpc (discussed in section 1.1.2.3.2). Although the initial dopaminergic cell death occurring in PD is not attributed to an hyperactive STN, subthalamic disinhibition ensuing the onset of nigrostriatal degeneration is thought to drive excess glutamatergic stimulation of the SNpc and hence increase the probability of excitotoxicity, creating a perpetuating degenerative cycle (Rodriguez *et al.*, 1998). For this reason, it is not surprising that the STN is receiving considerable interest in the pursuit to develop novel therapeutics. This has implications for symptomatic relief in PD, but more importantly to the work described in this thesis, potential to provide neuroprotection by suppressing the hyperactive STN to reduce a state of excitotoxicity.

#### **1.3.4 Dopaminergic denervation on the internal segment of the globus pallidus and the substantia nigra pars reticulata**

Under parkinsonian conditions alterations in the striatofugal system leads to profound changes in the output nuclei of the basal ganglia. As mentioned, the SNpr and the GPi receive increased glutamatergic transmission from the hyperactive STN in addition to decreased GABAergic transmission from the striatum and to an extent the GPe via the indirect pathway. Collectively, the net effect of these alterations in the basal ganglia is to increase GABAergic output from the SNpr and the GPi which has been supported experimentally by increased neuronal activity shown by increases in CO-1, GAD<sub>67</sub> and 2-DG expression in parkinsonian rodent and primate SNpr and GPi nuclei (Herrero *et al.*, 1996; Mitchell *et al.*, 1989; Vila *et al.*, 1997). Furthermore, in 6-OHDA-lesioned rodents there is an increase in the proportion of burst-type firing in SNpr neurones, an effect which is lost by kainic acid lesioning of the STN (Tseng *et al.*, 2000). In addition, 6-OHDA lesions of the SN in rodents, has been shown to increase GPi expression of GAD<sub>67</sub> mRNA, where STN lesions have also been shown to inhibit this increase in GAD<sub>67</sub> expression (Billings & Marshall, 2004). Furthermore, hyperactivity of the GPi has been shown in patients undergoing pallidotomy, where doses of apomorphine sufficient to reverse akinesia and tremor reduced the activity in the GPi by approximately 50% (Lozano *et al.*, 1998). Taken together, these results support the notion that an increase in GPi/SNpr firing underlies

the motor symptoms of striatal dopamine denervation. Consequently, this increased activity in the output nuclei leads to increased inhibitory GABAergic stimulation of the thalamic relay nuclei and brainstem nuclei such as the PPN. It is thought that the resultant reduction in thalamocortical feedback primarily underlies the akinesia/bradykinesia, rigidity and tremor in PD (Lang & Lozano, 1996), whilst the suppression of brainstem nuclei such as the PPN, may lead to gait and postural disturbances (Pahapill & Lozano, 2000).

Despite the profound advances in our understanding of the complex interplay within connections of the basal ganglia and proposed parkinsonian model (figure 1.2), there remains an element of caution owing to some inconsistencies from clinical data. For example in the model described, a surgical thalamotomy should negatively impact motor symptoms by reducing thalamocortical feedback, however there is little suggestion of this based on clinical findings. In addition, the GPe (thought to be hypoactive), may revert to a burst-type firing pattern with no net change in activity in the parkinsonian brain of primates and PD patients. Therefore this current model is unable to predict alterations in basal ganglia firing patterns and no doubt underlies these inconsistencies that have arisen (Lang & Lozano, 1996). Thus predicting the effects of dopamine denervation and therapeutic interventions within this elaborate network of the basal ganglia may not always reveal an expected outcome, which must be an important consideration in the pursuit of novel therapeutic interventions.

## **1.4 Animal models of Parkinson's disease**

Animal models are an important aid to investigate the pathogenic mechanisms and therapeutic strategies in human disease. Through the use of animal models, the striatal dopamine deficiency was associated with symptoms of PD and L-DOPA was first used to compensate for striatal dopamine loss (Carlsson *et al.*, 1957). Prolonged use of L-DOPA however, commonly results in debilitating, dyskinesias (as previously discussed in section 1.4.1.1) and fails to address the progressive degeneration limiting its usefulness in the long-term. Moreover, the pathology of PD is poorly understood even to this day. Thus, it is of great importance to develop animal models to improve understanding into the pathogenesis of PD and to aid the discovery of novel therapeutics. As such, no single model is likely to be suitable for all studies and therefore numerous animal models have been characterized to selectively simulate the pathogenic, histological, neurochemical and clinical features of PD. These are summarised in table 1.1.

### **1.4.1 Pharmacological models**

#### **1.4.1.1 The Reserpine model**

The reserpine-treated rodent was one of the earliest animal models to be employed in PD research. In 1957 Carlsson *et al.*, reported that systemic administration of reserpine causes depletion of brain catecholamines leading to an akinetic state in rabbits. These findings led to the hypothesis, later confirmed in humans (Hornykiewicz., 1966), that the motor symptoms of PD result from a depletion in striatal dopamine (Bernheimer *et al.*, 1973). Furthermore this model was instrumental in first demonstrating the therapeutic efficacy of what still remains the gold standard treatment for PD, L-DOPA. The reserpine-treated mouse or most commonly rat has since become established as a robust screen for potential symptomatic efficacy of new drugs in PD.

Model	Symptoms	Pathological characteristics	Pathogenic relevance	Application	Limitations
<b>Reserpine</b>	Akinesia, rigidity	None	Pharmacological depletion of dopamine, 5-HT and NA.	Preclinical testing to improve symptoms	No nigral degeneration; reversible loss of monoamines
<b>Haloperidol</b>	Muscle rigidity, catalepsy	None	Pharmacological depletion of dopamine, also 5-HT and NA? Elevation in extracellular glutamate	Preclinical testing to improve symptoms	No pathological characteristics associated with PD; reversible
<b>6-OHDA</b>	Unilateral: rotations following apomorphine/ amphetamine; Bilateral: akinesia	Decrease in striatal TH, dopamine; increase in glutamate release; degeneration of TH-positive cells in SNpc	Oxidative stress; inhibition of complex I and IV of mitochondria	Preclinical testing of therapies to improve symptoms: screen pharmacological and genetic neuroprotective therapies	Acute: usually unilateral (hemiparkinsonian); no Lewy body formation; requires intracerebral injection
<b>MPTP</b>	Akinesia, rigidity, tremor in some species	Decrease in striatal TH; degeneration of TH-positive cells in SNpc; some loss of locus coeruleus neurons; $\alpha$ -synuclein aggregation?!	Chronic oxidative stress; chronic inhibition of mitochondrial complex I	Screen pharmacological and genetic neuroprotective therapies	Generally acute; rapid cell death; variable lesion size
<b>Rotenone</b>	Akinesia, rigidity, tremor,	Decreased striatal TH immunoreactivity; degeneration of TH-positive cells in SNpc; some loss of locus coeruleus neurons; $\alpha$ -synuclein aggregation	Chronic environmental toxin; chronic oxidative stress; inhibition of mitochondrial complex I; inhibition of proteasomal activity	Screen potential neuroprotective pharmacological and genetic therapies	High mortality rate; intrinsic resistance in some cases
<b>Paraquat-Maneb</b>	Decreased locomotor activity, hunched posture	Decreased striatal TH immunoreactivity	Environmental toxins/pesticide exposure; oxidative stress; inhibition of mitochondrial complex I and III	Screen pharmacological and genetic neuroprotective therapies	Minimal degree of cell death in SNpc; variable loss of striatal dopamine content; relatively high mortality;
<b>Proteasomal inhibitors</b>	Bradykinesia, rigidity, tremor, postural instability	Decreased striatal TH immunoreactivity, dopamine; degeneration of TH-positive cells in SNpc; proteinous inclusions related to Lewy bodies	Impairment ubiquitin proteasomal system; oxidative stress	Screen pharmacological and genetic neuroprotective therapies	Difficult to reproduce; variable
<b>Genetic</b>	Decreased locomotor activity, hunched posture	Decreased striatal TH immunoreactivity, dopamine	Inhibition to mitochondria; impairment to ubiquitin proteasomal system	Current utility in assessing contribution of gene mutations in pathogenesis of PD; potential to screen pharmacological neuroprotective therapies	No nigral degeneration; compensation following gene knock out

**Table 1.1: Key characteristics of current animal models of Parkinson's disease**

Reserpine (usual dose of 4-5 mg/kg s.c.) works by inhibiting the vesicular monoamine transporter, VMAT2. This leads to loss of storage capacity and hence depletion of brain (and peripheral) monoamines including noradrenaline and 5-HT as well as dopamine. Whilst this lack of selectivity for dopamine was once considered a drawback of the reserpine model to accurately reflect the biochemistry of PD, the subsequent realisation that noradrenergic and serotonergic systems are also affected in PD (Jellinger, 1991) argues in favour of the reserpine model being a relatively good mimic of the disease biochemistry. Most attention nevertheless has focused on the dopaminergic deficit, where reserpine has been shown to produce an approximately 85% loss of dopamine in the SNpc and greater than 95% dopamine depletion in the striatum (Heeringa & Abercrombie, 1995). Reserpine also induces changes in additional basal ganglia nuclei, where firing in the STN is increased by approximately 50% (Robledo & Feger, 1991), mimicking the increase in activity observed in PD. Whilst the reserpine model mimics major components of the biochemistry of PD and induces akinesia and rigidity that reflect clinical features of the disease, there is no nigral dopaminergic cell degeneration so the model is restricted to assessing novel approaches to symptomatic treatment. However, within this framework, the reserpine-treated rat has proven very useful at predicting the efficacy of both dopaminergic (pramipexole, L-DOPA) and non-dopaminergic drugs (muscarinic agonists such as benztropine). These findings highlight the strong predictive validity of the reserpine-treated rat and justify its maintained position as a key model of choice for early preclinical stages of drug discovery programmes.

#### **1.4.1.2 The Haloperidol model**

The other pharmacological model of PD is the haloperidol-treated rat which acts to antagonise dopamine D<sub>2</sub> and to a lesser extent D<sub>1</sub> receptors in medium spiny neurons. The resultant block of striatal dopamine transmission leads to abnormal downstream firing within the basal ganglia circuits resulting in muscle rigidity and catalepsy within 60 min of haloperidol (0.5-5 mg/kg i.p.) injection (Sanberg, 1980). Whilst rigidity is a characteristic of PD, providing a degree of face validity to this model (similarity in symptoms), catalepsy is not directly associated with this degenerative disorder. However, catalepsy may be likened to the instability of patients to initiate

movements and so could be considered a worthwhile measure. Initial evidence suggested haloperidol was a poor biochemical mimic of PD, but recent evidence demonstrating haloperidol reduces striatal dopamine along with 5-HT and noradrenaline (Kulkarni *et al.*, 2009) may suggest otherwise. In addition, elevated levels of extracellular glutamate likely attributed from an increase in STN activity, have been reported in the entopeduncular nucleus following haloperidol injection supporting the construct validity (similar pathogenesis) of this model.

In common with the reserpine model, the haloperidol model fails to display any of the characteristic pathology associated with PD, so its use is again limited. Nonetheless, it remains a popular choice for assessing the potential symptomatic efficacy of novel non-dopaminergic agents including mGlu<sub>4</sub> positive allosteric modulators (PAMs) and adenosine A<sub>2A</sub>/A<sub>1</sub> antagonists (Niswender *et al.*, 2008; Neustadt *et al.*, 2009).

On a general note, whilst the pharmacological models have value in the discovery of symptomatic drugs for PD, they have serious limitations. Firstly, they remain transient, which hinders their long-term usefulness. In a condition such as PD, where drugs will be administered chronically, the need to assess the long-term symptom relief in animal models amenable to chronic dosing regimens is paramount. Secondly, these pharmacological models do not display any pathology of PD and consequently are no use in investigating novel strategies aimed at providing neuroprotective strategies.

## **1.4.2 Toxin-induced models**

### **1.4.2.1 6-OHDA lesion model**

Greater than 40 years ago Ungerstedt (1968) demonstrated that the hydroxylated analogue of dopamine, 6-hydroxydopamine (6-OHDA) led to degeneration of central monoamine neurones. Today the 6-OHDA-lesioned rat model is one of the most commonly used animal models of PD. Owing to its structural similarities to dopamine, following stereotactic injection into specific regions (due to its inability to cross the blood brain barrier), 6-OHDA is taken up into dopaminergic and

noradrenergic neurones via the high affinity dopamine and noradrenaline transporters (Blum *et al.*, 2001). Depending on the site of injection, 6-OHDA is either taken directly in the cell body (nigral administration) or retrogradely transported to cell bodies (MFB and striatal administration), leading to the accumulation of the toxin and induction of cell death. 6-OHDA differs from symptomatic models of PD such as reserpine or haloperidol since it leads to irreversible degeneration within the neurones that have taken it up by their respective transporters. Cell death via 6-OHDA is thought to occur by several mechanisms, including the production of reactive oxygen species (Kumar *et al.*, 1995), inhibition of complexes I and IV of the mitochondrial respiratory chain (Glinka & Youdim, 1995), a reduction of striatal antioxidants such as glutathione and superoxide dismutase (Betarbet *et al.*, 2002) and more recently activation of pro-apoptotic proteins, Bax and p53 upregulated modulator of apoptosis (PUMA) (Gomez-Lazaro *et al.*, 2008). All of these mechanisms, apart from the induction of apoptosis promote cell death by oxidative stress and mitochondrial dysfunction as discussed in detail in section.1.1.2.3.1. The 6-OHDA model also mimics many of the biochemical features of PD by reducing levels of striatal dopamine and TH. An important similarity with PD to this thesis is the increased firing of the STN (Hassani *et al.*, 1996; Hutchison *et al.*, 1998; Breit *et al.*, 2006) and parallel increase in glutamate levels which leads to alterations in firing of the basal ganglia output regions following a 6-OHDA lesion (Hutchison *et al.*, 1994). This has rendered the 6-OHDA model particularly useful to investigate glutamatergic agents which may provide neuroprotection by reducing hyperactivity within the STN and is discussed in greater detail in section 3.3.1.1.

The 6-OHDA model shares a common failing with many other animal models of PD in that it does not lead to Lewy body formation. Although the exact role of Lewy body formation remains to be established, drugs shown to reduce aggregate formation are considered a potential future strategy for treating PD. Nonetheless 6-OHDA displays robust degeneration of the nigrostriatal tract successfully mimicking this additional pathological hallmark of PD. Furthermore the extent of degeneration can be established post-mortem by assessing the reduction in various parameters in the lesion (ipsilateral) hemisphere, compared with the intact (contralateral) hemisphere such as levels of TH or DAT immunoreactivity in the SNpc and dopamine levels in

the striatum. Behavioural indices can also be taken to predict lesion size and provide additional measures of efficacy using potential symptomatic and neuroprotective agents, the latter of which is drawn upon considerably in this thesis and discussed in greater detail in section 3.3.1.2.

#### **1.4.2.2 MPTP-treated model**

MPTP is a commonly used toxin for inducing both rodent and primate models of PD based on its ability to induce persistent Parkinsonism in man (Davis *et al.*, 1979; Langston *et al.*, 1983). Subsequent studies in non-human primates have revealed the selective destruction of dopaminergic neurons of the nigrostriatal tract was the pathological basis behind the motor deficits observed (Burns *et al.*, 1983; Jenner *et al.*, 1989) which ultimately led to MPTP becoming the most widely used animal model to date.

The neurotoxic mechanism of action of MPTP is relatively well understood. Following systemic injection (usually i.p.), MPTP rapidly crosses the BBB, which once inside the brain is converted by MAO-B into MPP<sup>+</sup> (Chiba *et al.*, 1984). On release into the extracellular space, MPP<sup>+</sup> is taken up via DAT into dopaminergic neurons where cytoplasmic MPP<sup>+</sup> triggers the production of ROS leading to neurotoxicity (Javitch *et al.*, 1984). MPP<sup>+</sup> also inhibits complex I of the electron transport chain, leading to a reduction in ATP production and further generation of ROS. These factors combined are most likely responsible for initiation of cell death related signalling pathways such as p38 mitogen-activated kinase (Karunakaran *et al.*, 2008) and JNK (Saporito *et al.*, 2000).

The MPTP-treated mouse presents a clear advantage over the 6-OHDA model in being systemically active and not requiring skilled stereotaxic surgery to produce a lesion. The systemic injection also produces a bilateral degeneration of the nigrostriatal tract which is more reflective of that seen in PD. However, the MPTP model does have some clear disadvantages to the 6-OHDA model, particularly in terms of reproducibility and potential to investigate behavioural measures. Mice are far less sensitive to MPTP than primates, therefore high doses are often required



which can lead to higher mortality. The handling of MPTP also poses a risk to researchers and therefore extreme care must be taken to reduce exposure when handling the toxin and biological waste products from the treated animals (Przedborski *et al.*, 2001). Variability in lesion size is also an issue, whereby strain of mice, age, gender and weight, have all been shown to influence the reproducibility of an MPTP lesion. On a separate note, controversy still surrounds the issue of whether MPTP treated mice develop Lewy body-like inclusions. One of the first studies to assess this possibility revealed chronic treatment of MPTP for a period of 24 weeks, led to several of the remaining TH-positive cells in the SNpc containing  $\alpha$ -synuclein and ubiquitin-containing inclusions, although these did not resemble classical Lewy bodies found in the disease (Meredith *et al.*, 2002). Later studies have since failed to corroborate these findings using the same treatment regimen (Fornai *et al.*, 2005) or following multiple-acute or sub-chronic paradigms (Shimoji *et al.*, 2005). Further investigation is certainly required before this model can be used for assessing agents that may prevent aggregate formation. The MPTP model in mice has been able to predict the efficacy of non-dopaminergic agents such as the A<sub>2A</sub> antagonist, istradefylline (Shiozaki *et al.*, 1999), therefore this model would be expected to predict the ability of agents to provide protection against the degeneration in the MPTP-treated primate, given they share a common inducer.

### **1.4.3 Pesticide-induced models**

#### **1.4.3.1 Rotenone model**

On finding MPTP produced nigrostriatal tract degeneration through the targeting of mitochondrial complex I, the search for additional mitochondrial toxins to model PD led to the emergence of the rotenone model. Like MPTP, the insecticide rotenone is highly lipophilic, so it readily crosses the BBB and diffuses into neurons where it accumulates within the mitochondria and inhibits complex I. The cause of toxicity is thought to be a result of the ensuing production of ROS and subsequent glutathione depletion rather than the direct reduction in ATP. Furthermore extensive microglial activation observed in both the SNpc and striatum following rotenone infusion (Sherer *et al.*, 2003) is consistent with the inflammatory characteristic found in

idiopathic PD (Gerhard *et al.*, 2006; Whitton, 2007) further supporting the validity of this model. Additional support of the rotenone model is offered by a recent finding demonstrating rotenone inhibits proteasomal activity (Wang *et al.*, 2006c). Unfortunately, in addition to its central toxicity, rotenone shows a high degree of systemic (predominantly cardiovascular) toxicity that leads to high mortality rates (~30% of animals). There also appears to be an intrinsic resistance of some rats to rotenone, with some studies reporting as few as 50% displaying degeneration (Betarbet *et al.*, 2000), requiring additional animals.

Administration of rotenone, typically 2-3 mg/kg i.p. daily, demonstrates a dose-dependent loss of striatal TH and dopamine up to a period of 2 months accompanied with a reduction in locomotor activity and marked catalepsy that is reversed by L-DOPA (Alan & Schmidt, 2004). The pattern of cell death mirrors that seen in idiopathic PD, where greater cell loss is apparent in the ventral tier of the SNpc with relative sparing of the VTA and only a degree of degeneration of noradrenergic neurones in the locus coeruleus. Furthermore  $\alpha$ -synuclein and ubiquitin-positive Lewy body-like intracytoplasmic inclusions have been reported with rotenone (reviewed in Duty, 2011) lending support to the construct validity of this model, although there has been some difficulty in reproducing these findings.

Whilst the variable nature of rotenone has been useful to examine aspects related to the pathophysiology of PD, the current model does not provide a robust platform to assess the effects of symptomatic drugs. Indeed, L-DOPA and apomorphine to date remain the only clinically available drugs that have shown to reverse locomotor deficits in the rotenone model (Alan & Schmidt, 2004). However, worthy of mention is that selegiline and pramipexole, both of which were examined for disease modifying potential in clinical trials but then failed due to lack of efficacy, did protect against rotenone-induced degeneration (Saravanan *et al.*, 2006; Inden *et al.*, 2009), suggesting the rotenone model may show promise for selecting the efficacy of potential neuroprotective agents.

#### 1.4.3.2 Paraquat and Maneb

Exposure to the herbicide 1,1'-dimethyl-4,4'-bipyridinium (paraquat) or the fungicide ethylene-bis-dithiocarbamate (Maneb) has been associated with an increased incidence of PD (Ascherio *et al.*, 2006; Costello *et al.*, 2009). As a result, attempts have been made to model PD using both these agents. Following systemic injection in mice, paraquat crosses the BBB, which once inside cells, leads to both indirect mitochondrial toxicity via redox cycling and also direct inhibition of complex I whilst Maneb preferentially inhibits complex III following entry into the brain. Paraquat and Maneb have been shown to produce enhanced toxicity when combined (Thiruchelvam *et al.*, 2000), likely a result of Maneb increasing the brain concentration and reducing clearance of paraquat (Barlow *et al.*, 2003). Also since both pesticides are used in the same geographical regions, human exposure to one of these pesticides alone is unlikely, providing a clear rationale for combining their administration in order to produce an animal model of PD. Indeed, combined paraquat and Maneb exposure in both mice and rats led to greater denervation of the dopaminergic system than either chemical alone (Thiruchelvam *et al.*, 2000). In most cases in mice, this is typically accompanied with motor deficits manifest as hunched posture and a decline in locomotor activity. Some treated animals however, suffer from progressive weight loss and respiratory pathology leading to quite high mortality. Furthermore this model has been criticised for its minimal degree of cell death and variable loss (if any) of striatal dopamine content. There currently remains no evidence for presence of inclusions, which taken together has limited the use of this model in drug discovery programmes. Nonetheless, the paraquat and Maneb models do provide credence to the theory that environmental toxins and pesticides might have a role in PD pathogenesis.

#### 1.4.4 Proteasomal inhibitor models

Interest in Lewy body formation was rekindled by the discovery of mutations in  $\alpha$ -synuclein being responsible for rare forms of familial PD. When Lewy bodies were shown to be intensely immunoreactive for  $\alpha$ -synuclein and also to contain nitrated forms of the protein, the failure of protein metabolism in PD was proposed as the root

cause to the neuronal loss (Spillantini *et al.*, 1998; Duda *et al.*, 2000). The following discovery of two mutations in familial PD, UCHL1 and Parkin, both of which affect ubiquitin proteasomal function (see section 1.1.2.1), provided additional weight to this possibility. Therefore, when a reduction in proteasomal catalytic activity in the SN in PD was demonstrated, attempts to use this as a means of developing a new model began (McNaught & Jenner, 2001). Proteasomal inhibitors such as lactacystin, PSI and epoximycin, have since been shown to selectively kill dopaminergic cells in culture and further induce nigrostriatal degeneration following intranigral injection, reduce striatal dopamine content and induce motor deficits in rats (McNaught *et al.*, 2002a). More encouragingly, a report that systemic administration of PSI/epoximycin could reproduce many components of PD as affected in man, suggested this model may have strong face value (McNaught *et al.*, 2004).

Unfortunately these early findings have proven difficult to reproduce and reports of failure in mice, rats and primates have since emerged. Recent investigations have shown that the dose of PSI is critical with optimal dosage levels above which toxicity decreases (Bukhatwa *et al.*, 2010). Differences between routes of administration with effects observed following subcutaneous and oral administration but not intraperitoneal injection have also contributed to the variability in response to PSI. These factors have all been a frustrating hindrance since PSI could potentially be a valuable model of PD in which to test neuroprotective strategies, yet nothing has so far appeared in the literature.

#### **1.4.5 Genetic models**

There have been major advances in determining the underlying gene defects in familial PD that have led to numerous attempts to produce transgenic models of PD in mice. The gene products identified have shown both commonality with mechanisms of neuronal cell death in sporadic PD, via mitochondrial dysfunction ( $\alpha$ -synuclein, PINK1, DJ-1 and LRKK2) and alterations in protein folding and metabolism ( $\alpha$ -synuclein, parkin UCHL1). Indeed, attempts to produce knockouts, transgenics and over-expressers have revealed a variety of abnormalities. For example LRKK2 transgenic mice demonstrate dopaminergic dysfunction along with

behavioural deficits that are L-DOPA responsive although no noticeable nigral degeneration has been reported (Lin *et al.*, 2009). Furthermore PINK1, DJ-1 and Parkin knockouts all demonstrate mitochondrial dysfunction and reduced evoked striatal dopamine release yet also failed to replicate nigral pathology. The reason for this failure remains largely a mystery although one suggestion is that gene defects reported may not themselves be the cause of familial PD, but rather operate through epigenetic effects that allow, for example the expression of effects of ‘silent genes’ which then initiate dopaminergic degeneration. An additional explanation may be that when genes are knocked out or over-expressed embryonically, compensatory mechanisms may occur in the dopaminergic system to effectively mask the effects of genetic manipulation. This would imply a shift to conditional knockouts or viral vector-mediated delivery of transgenes may yield improved models for the future. Although the genetic mice models have not yet contributed to drug discovery for PD, it is possible that with further optimization, such models will one day contribute in this way. However at this moment in time, the current genetic models have most utility in investigating how the gene mutations associated with PD may contribute to disease pathogenesis.

## **1.4 Current therapeutic strategies for Parkinson's disease**

### **1.4.1 Dopamine replacement therapy**

To date the most successful attempts at pharmacotherapy for PD have focused largely on the symptomology. As the underlying pathology of PD involves loss of dopaminergic innervation within the striatum, these therapies have focused on either replicating the actions of dopamine at dopaminergic receptors or dopamine replacement strategies. Currently, dopaminergic strategies remain the primary treatment option in the management of PD.

#### **1.4.1.1 L-DOPA**

The current gold standard for PD therapy is treatment with the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA). Unlike dopamine, L-DOPA readily crosses the blood brain barrier (BBB) where in the brain it is converted by dopa decarboxylase into dopamine by the remaining dopaminergic neurons, thus replacing lost dopamine from the nigrostriatal projections. L-DOPA is typically administered concomitantly with a peripheral L-aromatic amino acid decarboxylase (L-AAAD) inhibitor such as carbidopa or benserazide to prevent conversion of L-DOPA to dopamine in the periphery. Carbidopa can increase the amount of L-DOPA entering the brain from 1 to 10% of the administered dose enabling a 10-fold decrease in the therapeutic dose required. Furthermore, by reducing peripheral dopamine, there are improvements in peripheral side effects such as hypotension in addition to nausea and vomiting which are a result of effects mediated by dopamine on the medullary vomiting centre (which is not protected by the BBB) (Jankovic, 2002). In addition, monoamine oxidase B (MAO-B) inhibitors such as selegiline are often given as an adjunct therapy to increase the effectiveness of L-DOPA by blocking the breakdown of dopamine in the brain, further enabling the use of reduced doses of L-DOPA.

Initial treatment with L-DOPA is very effective with patients demonstrating significant improvements in rigidity and akinesia, and in some cases complete restoration to normal. However, L-DOPA does not halt the progression of the disease,

nor does it pose any therapeutic efficacy to non-motor symptoms. Furthermore with persistent L-DOPA treatment, patients begin to experience end of dose deterioration and an emergence of motor fluctuations- the wearing 'on' and 'off' phenomena. In these circumstances rigidity and bradykinesia may suddenly appear, as well as the gradual initiation of excess involuntary movements of choreic, dystonic nature known as dyskinesias. Although a reduction in dosage of L-DOPA reduces the incidence of these motor complications, bradykinesia and rigidity can resume, and the therapeutic window for L-DOPA becomes progressively smaller with time. This phenomenon affects approximately 40% of PD patients after approximately 5 years of treatment with L-DOPA, increasing to around 95% after 10 years (Ahlskog & Muenter, 2001). Several mechanisms have been proposed to underlie the motor complications induced by L-DOPA and will be discussed in more detail below.

With ongoing degeneration in PD it has been suggested that the site of L-DOPA decarboxylation changes from striatal terminals to non-dopaminergic sites, such as interneurons and glia. This shift in decarboxylation site may contribute towards the development of motor complications such as dyskinesias (Guigoni *et al.*, 2005). In addition to this putative shifting of L-DOPA decarboxylation, the unrelenting neurodegeneration leads to more pulsatile dopamine receptor stimulation in contrast to the expected continuous stimulation in normal conditions. This may attribute to the cyclic blood plasma levels associated with oral administration of L-DOPA, in addition to the ongoing degeneration of the nigrostriatal tract in which the effects of endogenous dopamine progressively deteriorate. It has been proposed that pulsatile stimulation can alter the electrophysiological firing patterns of basal ganglia nuclei, particularly the STN and contribute to the emergence of the on-off phenomena and dyskinesias (Olanow *et al.*, 2004). Indeed, both short acting D<sub>1</sub> agonists such as SKF 82958 and D<sub>2</sub> agonists given to MPTP-treated monkeys by repeated pulsatile administration, gives rise to increased levels of dyskinesia, when compared to continuous receptor stimulation brought about by dopamine agonists with longer half-lives (Calon *et al.*, 2000; Jenner, 2000). Furthermore, concomitant administration of catechol-O-methyl transferase (COMT) inhibitors such as tolcapone and entacapone with L-DOPA has been shown to reduce the on-off phenomena (Brooks & Sagar, 2003). These compounds prevent the peripheral conversion of L-DOPA into its

metabolite 3-O-methyldopa (3-OMD) which accumulates due to its long half-life (>15 hours) and competes with L-DOPA for transport across the BBB. Thus the use of COMT inhibitors increases the bioavailability of L-DOPA in the brain and improves motor complications by resuming continuous dopamine receptor stimulation within the striatum.

An additional explanation for the motor complications relating to L-DOPA treatment, particularly in the cause of dyskinesias is dopamine receptor 'supersensitivity'. It is thought long-term dopamine receptor stimulation may lead to supersensitivity and consequent imbalance between the direct and indirect pathways (Obeso *et al.*, 2000). Indeed in parkinsonian conditions, supersensitivity to dopamine may be explained by the observed increase in striatal expression of D<sub>2</sub> receptors despite the continuing degeneration of the nigrostriatal system. In further support of this notion is evidence for increased downstream signalling of D<sub>1</sub> receptors, at the level of the G-protein, through extracellular signal-related kinase 1/2 (ERK1/2) and mitogen-activated protein kinase (MAPK) pathways, which may also be a result of receptor supersensitivity (Hurley & Jenner, 2006). Thus, although the exact mechanism underlying the initiation of motor complications following treatment with L-DOPA remains to be determined, it would appear these effects may implement some, if not all of the mechanisms described.

Whilst dopamine replacement therapy with L-DOPA has been shown to be highly efficacious in PD patients, some controversy remains about its possible toxicity. Both L-DOPA and dopamine are known to undergo auto-oxidation which can lead to increased levels of reactive oxygen species and free radicals, in addition to a reduction in antioxidants (described in section 1.1.2.3.1). These agents can then lead to lipid peroxidation and damage to DNA, possibly resulting in apoptosis. A number of *in vitro* studies have reported L-DOPA mediated toxicity on dopaminergic cell cultures (Michel & Hefti, 1990; Pardo *et al.*, 1993; Steece-Collier *et al.*, 1990) which could be inhibited by antioxidants implying oxidative stress is a likely mechanism (Ziv *et al.*, 1997). Nonetheless, there are many criticisms of these studies such as the lack of supporting glial components and the absence of the antioxidant, ascorbate, in addition to the high doses of L-DOPA used in these studies. Indeed in the presence of



ascorbate and the glial cell component, L-DOPA was no longer found to be toxic to cultured dopaminergic neurons (Mena *et al.*, 1997). Moreover, there is almost no *in vivo* data to support the hypothesis that L-DOPA is toxic. Examples of such studies include a 6-month treatment study with L-DOPA in 6-OHDA-lesioned rats which revealed no alterations in dopaminergic cell numbers with respect to vehicle-treated animals in the lesioned hemisphere (Murer *et al.*, 1998). Furthermore in the clinic, chronic L-DOPA treatment for tremor and dystonia revealed no toxic effects on dopaminergic neurones in the SNpc (Rajput *et al.*, 1997). The neurotoxicity of L-DOPA therefore remains unproven in both animal models and in the clinic. Despite the obvious motor complications and extensive efforts to reveal more suitable alternatives, L-DOPA, has remained the gold standard in PD treatment for more than 40 years.

#### **1.4.1.2 Dopamine agonists**

Dopamine agonists form the main therapeutic alternative to L-DOPA in PD. Current dopamine agonists such as pramipexole, ropinirole and cabergoline have been designed with longer half lives when compared to L-DOPA, in an attempt to minimise the pulsatile stimulation thought to trigger dyskinesias, whilst maintaining antiparkinsonian efficacy. Indeed, such compounds have been demonstrated to control the motor symptoms of PD for up to 5 years with a lower incidence of dyskinesias when compared to L-DOPA therapy (Rascol, 2000). This has been confirmed in a study in which administration of L-DOPA to MPTP-treated primates led to the rapid onset of severe dyskinesia, yet in contrast, ropinirole produced only mild dyskinesia (Jackson *et al.*, 2007). Accordingly treatment of PD with longer lasting dopamine agonists is now thought to be an effective early monotherapy. D<sub>2</sub>-like (D<sub>2</sub>, D<sub>3</sub> & D<sub>4</sub>) inhibitory receptors are particular preferred to produce anti-parkinsonian effects, in order to target the profound effect of the indirect pathway, therefore most current dopamine agonists demonstrate affinity at these receptors. Indeed, pramipexole has no affinity for the D<sub>1</sub> receptor, high for D<sub>2</sub> and higher still for the D<sub>3</sub> receptor (Jenner, 2002). Furthermore, partial agonists have also been considered to reduce dopamine-mediated side effects such as hallucinations and psychosis which are associated with the modulation of healthy mesolimbic and

mesocortical pathways. For example, dihydroergocriptine a partial agonist at D<sub>1</sub> receptors and full agonist at D<sub>2</sub> receptors has shown antiparkinsonian efficacy in humans (Albanese & Colosimo, 2003). It is thought the side effect profile with these agents is reduced by exerting maximum effect in the denervated striatum, but a lesser effect when competing with endogenous dopamine in healthy normal regions. Considerable evidence would also suggest dopamine agonists may be neuroprotective (reviewed by Schapira, 2003). In positron emission tomography (PET) imaging studies using [<sup>18</sup>F]-fludopa and [<sup>123</sup>I]-beta-CIT, a slower loss of striatal dopamine storage in PD patients initially treated with ropinirole and pramipexole was observed compared to L-DOPA (Whone *et al.*, 2003). However it is important to point out no control group was available therefore we cannot be certain of a neuroprotective effect here since this may also be a result of toxicity caused by L-DOPA.

A recent advance in the treatment of PD is the development of slower release agents and use of transdermal patches to administer dopamine agonists which enables stable continuous release of drug throughout the day. One of these, rotigotine, is a mixed D<sub>1-3</sub> receptor agonist which has been developed as a transdermal patch and has shown efficacy in numerous pre-clinical models of PD (Jenner, 2005). In the clinic rotigotine is well tolerated in humans providing an effective monotherapy for early PD which also decreases 'off' time in subjects with advanced PD which cannot be controlled with L-DOPA (Lewitt *et al.*, 2007; Watts *et al.*, 2007).

## **1.4.2 Non-dopaminergic pharmacotherapy**

### **1.4.2.1 Cholinergic drugs**

Before the discovery of L-DOPA, cholinergic drugs were one of the first available options for therapy in PD intended to correct the imbalance between dopamine and acetylcholine (ACh) by negatively regulating cholinergic striatal interneurons. Currently a number of anticholinergic drugs are available such as trihexylphenidyl and benztropine. Whilst proven to be effective in reducing tremor in PD, these drugs offer few improvements in additional motor symptoms and are also associated with adverse cognitive side effects (reviewed in Katzenschlager *et al.*, 2003). In an attempt

to address this issue the more recent cholinesterase inhibitors, rivastigmine and donepezil (which were originally marketed for Alzheimer's disease) have been found to improve cognition in PD patients with dementia, although benefits on motor function were at best mild (Emre *et al.*, 2004; Fabbrini *et al.*, 2002; Leroi *et al.*, 2004). Thus, owing to the mild antiparkinsonian efficacy of cholinergic drugs, they are rarely used as a monotherapy, rather favoured in a combination with a dopamine replacement agent to selectively address clinical symptoms.

#### **1.4.2.2 Amantadine**

Amantadine is an anti-viral drug for the prophylaxis of influenza and was discovered to have antiparkinsonian efficacy accidentally. Although amantadine was originally thought to increase dopamine release likely via a mechanism on dopamine transporters or the receptors themselves, further investigation into the pharmacology has shown amantadine is an NMDA receptor antagonist (Starr, 1998). Consequently, amantadine may reduce the effects of hyperactivity in the glutamatergic subthalamonigral pathway and the potential of targeting such ionotropic glutamate receptors is discussed in greater detail in section 1.5.2.1 Amantadine itself however, has demonstrated only mild antiparkinsonian effects although it has been shown to be effective in reducing the prevalence of L-DOPA-induced motor complications (Verhagen *et al.*, 1998). Therefore, amantadine is primarily used as combination therapy with L-DOPA in patients who do not tolerate optimal doses, where it may help reduce motor fluctuations.

### **1.4.3 Future therapeutic prospects**

#### **1.4.3.1 Neurotrophic factors**

A reduction in neurotrophic factors such as brain-derived neurotrophic factor (BDNF) has been identified in PD (Howells *et al.*, 2000), therefore replacement of such factors has been suggested to hold therapeutic potential. In fact several neurotrophic factors have been examined in human clinical trials. These include glial-derived neurotrophic factor (GDNF), a potent growth factor that supports the survival of

dopaminergic neurones and has shown to be neuroprotective in animal models of PD (Eslamboli *et al.*, 2005; Kordower *et al.*, 2000). So far, focus has been on delivering GDNF primarily by direct infusion, which into MPTP-treated primates has shown to increase dopamine levels in the putamen by up to 2-fold resulting in improved motor function and decreased incidence of dyskinesias (Grondin *et al.*, 2002). A small open-label trial involving putamenal infusion of GDNF also demonstrated significant clinical improvements reporting a reduction in dyskinesias and increase in dopamine storage after 18 months determined by PET scans of [ $^{18}\text{F}$ ]-fluorodopa uptake (Gill *et al.*, 2003). However, a larger controlled study involving 34 PD patients was halted due to severe adverse effects in three cases and several other patients developing serum antibodies to GDNF (Lang *et al.*, 2006). A follow-up toxicology study in monkeys revealed infusion of GDNF showed cerebellar cell loss raising further concerns regarding the safety of GDNF infusions.

An alternative to direct infusion of trophic factors is delivery of these agents by gene transfer. Recent gene therapy efforts have used neurturin, a neurotrophic factor related to GDNF that also promotes dopaminergic cell survival in mid-brain dopaminergic cultures (Horger *et al.*, 1998). In addition, delivery of an adeno-associated viral (AAV-2) vector containing neurturin (CERE-120) protected against dopaminergic cell loss and improved motor function in MPTP-treated monkeys (Kordower *et al.*, 2006). Furthermore an open-label clinical study involving 12 patients with advanced PD, revealed promising findings, reporting off-medication UPDRS motor scores were significantly reduced and mean on time was increased at one year following injection (Marks *et al.*, 2008). However, data from open-label trials (in which there is no blinding to treatment) must be interpreted cautiously. Therefore the potential antiparkinsonian efficacy of neurotrophic factors has revealed some encouraging findings and continues to remain an area of high interest within the field.

#### **1.4.3.2 Antioxidants**

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>, ubiquinone), in serving as the electron acceptor for complexes I and II of the mitochondrial electron transport chain, is an antioxidant. Therefore the

potential of CoQ<sub>10</sub> to protect from mitochondrial dysfunction and/or oxidative damage in PD has been investigated. Bearing weight to this suggestion is a study that demonstrated following rotenone-induced toxicity *in vitro*, CoQ<sub>10</sub> and an additional antioxidant,  $\alpha$ -tocopherol reversed rotenone-induced toxicity (Sherer *et al.*, 2007). Numerous clinical trials have since been undertaken, in which the most promising revealed 16-month treatment with oral CoQ<sub>10</sub> facilitated a reduction in disability and slowed disease progression in mild PD (Shults *et al.*, 2002). Additionally CoQ<sub>10</sub> appeared to demonstrate no adverse effects and appeared to be well-tolerated, laying the passage for larger controlled trials which are now underway to probe the potential of antioxidants in PD therapy.

#### **1.4.3.3 Adenosine receptor antagonists**

Epidemiological studies have indicated that caffeine may reduce the incidence of PD, at least in men (Ascherio *et al.*, 2001; Ross *et al.*, 2000). As caffeine mediates its actions by antagonising adenosine receptors, this finding has led to interest in evaluating adenosine receptor antagonists as potential neuroprotective agents. In the striatum the adenosine A<sub>2A</sub> receptor can heterodimerize with the D<sub>2</sub> receptor to inhibit dopamine signalling (Ferre & Fuxe, 1992), whilst inhibition of the A<sub>2A</sub> receptor can promote dopamine function. The adenosine A<sub>2A</sub> antagonist ST1535 has been shown to potentiate a threshold dose of L-DOPA in both 6-OHDA lesioned rats and MPTP-treated marmosets suggesting that these receptors may offer symptomatic relief in PD (Rose *et al.*, 2006; Rose *et al.*, 2007). Furthermore, two small clinical trials using the A<sub>2A</sub> antagonist istradefylline (KW-6002) have demonstrated potential symptomatic effects in advanced PD (Bara-Jimenez *et al.*, 2003; Hauser *et al.*, 2003) where more recent research has suggested that A<sub>2A</sub> antagonists may also afford neuroprotection. Caffeine and istradefylline have both been shown to be neuroprotective in MPTP-treated primates, where caffeine at doses comparable to those of typical human exposure, attenuated MPTP-induced loss of striatal dopamine transported binding sites (Chen *et al.*, 2001). Indeed recently, caffeine (and related adenosine A<sub>2A</sub> receptor antagonists) has been identified by NIH-appointed Committee to Identify Neuroprotective Agents in Parkinson's disease (CINAPS) as a priority agent to be evaluated for neuroprotection in clinical trials.

## **1.4.4 Non-pharmacological therapy**

### **1.4.4.1 Stem cells and tissue transplantation**

Transplantation of dopaminergic tissues involving striatal infusions of autologous adrenal medullary cells, and later fetal ventral mesencephalon (VM) cells was pioneered by Swedish surgeons in 1982. Initially, these results were highly promising, although later double-blinded trials have failed to replicate these earlier findings. In one study, forty patients with severe PD were randomly assigned to receive transplants of fetal mesencephalic tissue or sham surgery and monitored for a year post-operation. This study found a significant benefit in UPDRS scores in patients under 60 although no improvements in older patients. Moreover, fibre outgrowth was present in 17 out of the 20 patients that underwent transplants, indicated by an increase in  $^{18}\text{F}$ -fludopa uptake using PET imaging (Tintner & Jankovic, 2002). However this approach has been limited by poor neuronal survival recorded following transplantation (in certain instances up to 90% loss of grafted material after one week post transplantation), limited tissue availability and lack of homogenous product. Therefore alternative sources of cells have been considered such as embryonic stem (ES) cells in the early stages of development.

Since stem cells are pluripotent, they can be isolated and expanded in culture to produce potentially large amount of uniform product. Early animal studies have compounded the potential of this approach, where a monumental study conducted by Bjorklund *et al.*, (2002) demonstrated following transplantation of undifferentiated mouse cells into the striatum of 6-OHDA-lesioned rodents, led to the proliferation of ES cells into differentiated dopamine neurones. In addition, this same study revealed ES- cell derived dopaminergic neurones were functional, demonstrating behavioural restoration of dopamine-mediated motor asymmetry (Bjorklund *et al.*, 2002). Consequently, ever since these early pre-clinical findings heavy investment has been poured into this area to determine if this approach offers the same potential in a clinical setting which may arguably offer the most promising hope to elucidate a 'cure' for PD. Despite the mass media attention, the current consensus is that there

are still considerable lengths to be achieved before this approach becomes a viable alternative for the management of PD.

#### **1.4.4.2 Surgical interventions**

Since the invention of the human stereotactic frame in the 1950s, there has been interest in surgical lesioning techniques to ameliorate symptoms of PD. With the advent of levodopa therapy in the 1960s and 70s, interest in surgical approaches for PD therapy waned. However, in the last decade enthusiasm has re-emerged, as a consequence of increased understanding of the pathophysiology of PD, coupled with advances in medical imaging and surgical techniques. Given the relative success of L-DOPA therapy, surgical approaches are generally reserved for those patients for whom the side-effects of L-DOPA therapy become untenable. Three main surgical targets have been evaluated, the thalamic nuclei, the GPi and the STN, with irreversible ablative procedures now being superseded by reversible deep brain stimulation (DBS). Bilateral DBS offers numerous advantages over lesioning since it is reversible, allows the levels of the stimulation to be adjusted and further reduces the likelihood of re-operation due to inadequate lesion volume. Bilateral DBS is also associated with decreased morbidity compared to bilateral lesioning (Tasker, 1997). Since thalamic stimulation has been shown to be highly effective in controlling tremor, yet shown limited effectiveness against other motor symptoms of PD, it is generally only recommended in a small number of cases demonstrating tremor-predominant disease (Walter & Vitek, 2004). In contrast, DBS of the STN or GPi has been shown to be highly effective in treating the majority of motor symptoms of PD, allowing reductions in L-DOPA dosage (and thus reducing incidences of dyskinesias), improving tremor, bradykinesia and gait disturbances (Krack *et al.*, 2000).

Of the two target areas, bilateral DBS of the STN is now thought to be the preferred target, showing greater motor improvements in ‘off’ medication states in addition to the greatest reduction in L-DOPA usage (Anderson *et al.*, 2005). Furthermore two summary reviews of all published human STN-DBS studies, revealed Unified Parkinson’s Disease Rating Scales (UPDRS) motor scores in the ‘off’ phase of

treatment were improved by 56% after 12 months and maintained at 49% after 5 years where both outcomes were compared to pre-operative off-medication scores. In addition these studies revealed average reductions in L-DOPA dosage by 56% and dyskinesia incidence by 69% (Hamani *et al.*, 2005; Kleiner-Fisman *et al.*, 2006).

Whilst highly effective in treating the motor symptoms of PD, STN-DBS surgery does have some limitations. The most serious relating to surgery is intercranial haemorrhage (3.9%), whilst adverse effects relating to stimulation include speech disorders (dysarthria, 9.3%), weight gain (8.4%) and depression (6.8%) however these latter effects can be reversed by altering stimulation parameters (Kleiner-Fisman *et al.*, 2006). An additional drawback is that STN-DBS is a procedure not widely available especially in less well developed countries due to the high labour costs and expensive equipment required. Therefore on the basis of positive results in STN-lesioned MPTP-treated primates (Guridi *et al.*, 1996), bilateral subthalamotomy has been proposed as a feasible alternative to DBS where needed. However, this procedure has the disadvantage of being irreversible and may also result in hemiballism. Despite these limitations, a report by Alvarez *et al.*, (2005) on the long term effects of bilateral subthalamotomy on 18 patients with advanced PD, revealed significant reductions in L-DOPA dosage (47%), L-DOPA-induced dyskinesias (50%), and “off” time (49.5%) 2 years post-operatively compared to pre-operative baseline levels. Adverse effects from the surgery were generally minimal and transitory in nature, e.g. dyskinesia lasting for only 3 months (17%), whilst motor improvements remained for 4 years (Alvarez *et al.*, 2005).

Since STN-DBS and subthalamotomy achieve similar outcomes there appears to be a paradox, with high frequency (100Hz) stimulation resulting in equivalent effects to that achieved by surgical ablation of the nucleus. As a result numerous mechanisms of action of STN-DBS have been proposed and will be discussed in detail here. The first hypothesis is that high frequency stimulation of the STN may eventually lead to a block in neurotransmission. This could be achieved by a depolarising block of neuronal transmission through inactivation of voltage-dependent ion channels, or through stimulation-induced neurotransmitter depletion and consequent synaptic failure (Breit *et al.*, 2004). An alternative hypothesis involves the role of the GPe in



STN-DBS. DBS of the STN may increase activity in the subthalamopallidal connections to the GPe – the main projection target of the STN. As a result this could activate inhibitory GABAergic projections of the GPe which as discussed previously form reciprocal connections with the STN and could potentially decrease STN hyperactivity, consequently reducing glutamate release in the basal ganglia output nuclei. Interestingly however, increased levels of GABA and not decreased levels of glutamate have been found in the SNpr following DBS in 6-OHDA-lesioned rats (Windels *et al.*, 2005). This could again be explained by increased activity in the subthalamopallidal projections and resultant overactivity of the GPe, which would then lead to increased activity in the GABAergic pallidonigral projections potentially underlying the increase in GABA in the SNpr. Hence the mechanism of STN-DBS may be heavily dependent on modulation via the GPe, either to dampen glutamatergic activity through inhibitory reciprocal connections to the STN or to increase GABAergic activity in the SNpr, both of which would normalise thalamocortical feedback.

A more recent proposal for the mechanism of action of STN-DBS has emerged through a better understanding of STN firing patterns in PD. In PD synchronised oscillatory activity in the range of 10-35 Hz often referred to as the  $\beta$ -band, is prevalent in the basal ganglia-thalamocortical circuits (Gatev *et al.*, 2006). Therefore ‘jamming’ of the aberrant firing pattern of STN neurones from  $\beta$ -band frequencies, by imposing firing patterns at higher frequencies, may relieve PD symptoms (Breit *et al.*, 2004). Indeed a study in PD patients, where the effects of frequency of STN-DBS were investigated, demonstrated that stimulation between 5-10 Hz and 20-25 Hz were associated with bradykinesia, whilst higher frequencies ( $> 100$  Hz) relieved bradykinesia (Fogelson *et al.*, 2005).

Clearly there are many benefits of both STN-DBS and subthalamotomy however there are still drawbacks to these approaches. These include cost, the limited availability and post-operative complications. Therefore attention has been drawn to establishing a pharmacological approach which may achieve comparable outcomes. Taking into account the positive outcomes of surgically targeting the STN in PD, a pharmacological alternative to decrease overactive glutamatergic innervations of the

basal ganglia output nuclei would have far more widespread application than surgical procedures. Owing to the glutamatergic nature of the STN and proposed hyperactivity in the parkinsonian brain, agents that can interfere with glutamatergic neurotransmission present a promising strategy for the potential development of novel antiparkinsonian therapies. This pharmacological possibility forms the major focus of this thesis.

## **1.5 Glutamatergic receptors and their potential in Parkinson's disease**

The pivotal role of glutamatergic neurotransmission in the pathogenesis of PD has been discussed extensively in section 1.3. Whilst it is widely accepted glutamatergic function is implicated in the pathogenesis of PD it is surprising there is currently only one drug (amantadine) on the market that modulates the glutamatergic system. In this section it becomes clear why this is the case, largely owing to the fact glutamate is the core excitatory neurotransmitter in the mammalian CNS. Therefore manipulation of this system is likely to have vast implications and widespread side effects. Consequently recent developments in modulating the glutamatergic system have involved a more subtle approach.

### **1.5.1 Glutamate transmission**

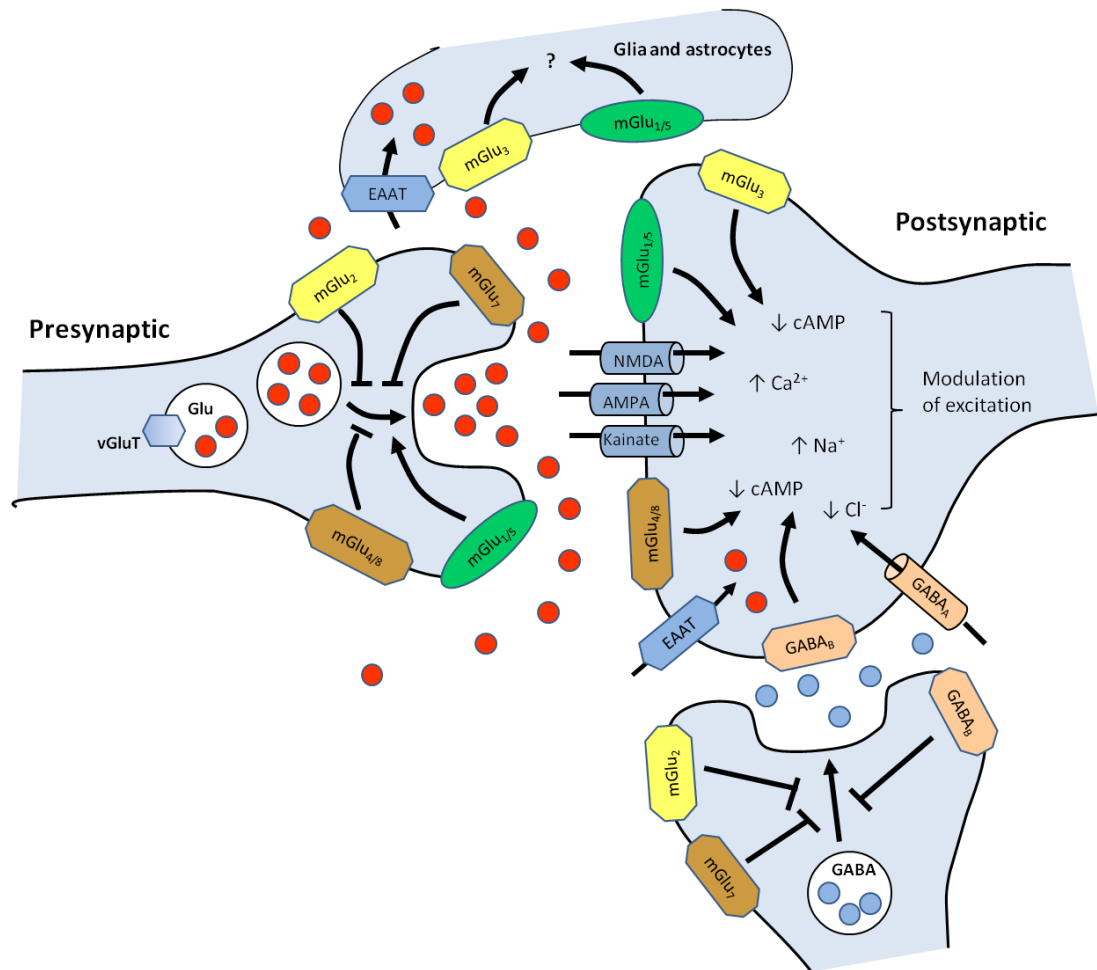
Following depolarisation-evoked glutamate release, synaptic accumulation of glutamate can lead to excitotoxicity (discussed in section 1.1.2.3.2) as well as receptor desensitisation and high background levels of glutamate, thus decreasing signal to noise ratio (Sinclair *et al.*, 2003). Since no extracellular enzymes exist that are capable of metabolising glutamate, its concentration is maintained at 1  $\mu$ M in the synaptic cleft by active re-uptake by five Na<sup>+</sup> and K<sup>+</sup>-driven transporters (Nicholls & Attwell, 1990) known as excitatory amino acid transporters (EAATs 1-5). Of these, GLT-1 (EAAT1) and GLAST (EAAT2) are exclusively expressed on astrocytes, whilst EAAT3 is expressed on both glutamatergic and non-glutamatergic neurones in addition to astrocytes and oligodendrocytes (Schousboe *et al.*, 2004). EAAT4 is expressed by Purkinje cells of the cerebellar molecular layer, whilst EAAT5 is restricted to Müller cells in the retina (Schousboe *et al.*, 2004). The majority of glutamate is taken up by astrocytes where it may be metabolised in two ways, either by being converted into glutamine by the ATP-dependent, glutamine synthase or by conversion to  $\alpha$ -ketoglutarate by glutamate dehydrogenase which is then metabolised to lactate in the Krebs cycle (Waagepetersen *et al.*, 2005). Both glutamine (which is non-toxic) and lactate are then exported from astrocytes and taken up into neurones via a low affinity Na<sup>+</sup>-independent system A transporter (Broer & Brookes, 2001).

This enables glutamine to be hydrolysed by glutaminase within neurones to form glutamate creating an intracellular concentration of approximately 10mM (Nicholls & Attwell, 1990). The reformed glutamate is then packaged into vesicles by an additional transporter system, the vesicular glutamate transporters (vGluT 1-3) which are  $\text{Na}^+/\text{K}^+$  independent transporters driven by  $\text{H}^+$ -ATPase. Finally, this repackaged glutamate is then set to be released into the synapse following propagation of an action potential generated in a  $\text{Ca}^{2+}$ -dependent manner where it may act on numerous pre- and post-synaptic glutamate receptors. The general synaptic localisation and function of glutamatergic receptors and transporters in summarised is illustrated in figure 1.3.

### **1.5.2 Glutamate receptors**

Glutamate receptors can be broadly divided into two main classes, referred to as the ionotropic and metabotropic families. Ionotropic glutamate receptors (iGlu) are ion-channel gated receptors where ligand-mediated activation leads to fast channel opening. In contrast, metabotropic glutamate receptors (mGlu) receptors are G-protein coupled receptors where activation leads to the initiation of secondary messenger and intracellular signalling cascades. Whilst the focus of this thesis lies on mGlu receptors, the potential in targeting these receptors arose from numerous experimental findings investigating a role for iGlu receptors. Therefore the ionotropic class of glutamatergic receptors are discussed here first.

Figure 1.3: Synaptic localisation and function of glutamatergic receptors and transporters.



**Figure 1.3.** Schematic illustration of the general synaptic localisation and function of glutamatergic receptors and transporters. The ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate subtypes, function predominantly to mediate fast receptor transmission and use-dependent changes required for synaptic plasticity. The vesicular transporters (vGluT) load glutamate into vesicles presynaptically whilst glial and astrocyte postsynaptic glutamate transporters (excitatory amino acid transporters, EAAT1-5) mediate the uptake of glutamate required to terminate synaptic transmission. The metabotropic glutamate receptors have a diverse synaptic localisation and function pre- and postsynaptically to modulate neurotransmitter release and postsynaptic excitability, respectively (figure adapted from Swanson *et al.*, 2005). GABA:  $\gamma$ -aminobutyric acid.

### 1.5.2.1 Ionotropic glutamate receptors

There are three main types of iGlu receptors which are named after the preferential ligand that activates them. These are  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate).

#### *1.5.2.1.1 AMPA receptors: Signal transduction, general distribution and therapeutic potential in PD*

AMPA receptors are responsible for the majority of fast excitatory transmission in the CNS, although they exhibit a much lower affinity ( $EC_{50}$  of 1mM) for the endogenous ligand glutamate, than NMDA receptors ( $EC_{50}$  of 2.3 $\mu$ M) (Hestrin, 1992; Patneau & Mayer, 1990). AMPA receptors comprise four subunits (GluR1-4) which are assembled as functional tetramers which can be both homomeric or heteromeric in composition (Rosenmund *et al.*, 1998). Upon activation, AMPA receptors mediate opening of an ion channel which is permeable to monovalent cations such as  $Na^+$  and  $K^+$ . The GluR2 subunit plays an important role in the determination of divalent cation permeability, where incorporation of this subunit is indicative of a low permeability to the resultant channel. (Hollmann *et al.*, 1991). However homomeric or heteromeric receptors composed of GluR1, GluR3 or GluR4, in the absence of GluR2, form ion channels that are permeable to  $Ca^{2+}$ ,  $Mg^{2+}$ , and  $Ba^{2+}$ . Further complexity is introduced by alternative splicing in the second extracellular domain with all AMPA subunits (GluR1-4) subunits being expressed as two alternate splice variants known as 'flip' and 'flop'. Flip variants exist predominantly pre-natally, whilst the flop variant is the major splice variant in adults. Flip and flop variants of AMPA receptors alter the sensitivity of these receptors to allosteric modulators in addition to regulating the extent of receptor desensitisation. In fact AMPA receptors respond rapidly to agonist application, displaying rapid (2-14 msec) desensitisation in response to full agonists, AMPA and glutamate, but not to partial agonists such as kainate (Kew & Kemp, 2005).

AMPA receptors are widely distributed throughout the basal ganglia where high levels of receptors containing the GluR1-3 subunits are found on striatal MSNs, the GPi and SNpc. Those containing the GluR4 subunit are expressed predominantly in the GPi, SNpr and on glial cells (Greenamyre, 2001). Therefore owing to their wide distribution and ability to mediate excitatory neurotransmission, AMPA receptors have been proposed as potential drug targets in the treatment of PD.

Antiparkinsonian effects of AMPA receptor antagonists both alone and in combination with dopaminergic therapies have been reported. For example, systemic administration of the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) has been shown to reverse reserpine-induced muscle rigidity (but not akinesia) in rats and motor deficits in MPTP-lesioned primates (Klockgether *et al.*, 1991). In addition, NBQX has been found to prolong the action of L-DOPA and decrease on-off phenomena in parkinsonian rats (Marin *et al.*, 2000). Interestingly, a separate study revealed NBQX improved the ability of L-DOPA to reverse motor deficits in SNpc-lesioned rats and primates (Loschmann *et al.*, 1991; Loschmann *et al.*, 1992; Wachtel *et al.*, 1992), suggesting AMPA receptor blockade may be a useful adjunct for improving the efficacy of L-DOPA treatment.

Owing to their role in excitotoxicity, as modulators of the initial depolarising stimulation and  $\text{Ca}^{2+}$  influx, AMPA receptors have also been suggested as potential targets to provide neuroprotection. These effects could be mediated through a number of mechanisms including; prevention of desensitisation of  $\text{Ca}^{2+}$ -impermeable GluR2 subunits, modulation of AMPA receptor binding partners such as postsynaptic density protein of 95 kDa (PSD-95), Glutamate receptor interacting protein (GRIP), and protein interacting with C-kinase (PICK) as well as directly modulating ion-channel function (reviewed by Jayakar & Dikshit, 2004). Indeed, AMPA receptor inhibition by the negative allosteric modulator, talampanel, has demonstrated neuroprotection in the striatum and cortex following ischemic brain injury in rodents (Erdo *et al.*, 2005). Furthermore, the authors suggest a reduction in glutamate-mediated excitotoxicity through selective blockade of  $\text{Ca}^{2+}$  permeable AMPA receptor ion channels as the predominant mechanism of action. Interestingly a separate study using AMPA positive allosteric modulators LY404187 and LY503430, has been shown to provide

protection against MPTP-induced toxicity in mice and lead to functional, histological and neurochemical improvements following 6-OHDA-lesioning in rodents (O'Neill *et al.*, 2005; O'Neill *et al.*, 2004). Despite increasing glutamate-induced calcium influx into HEK 293 cells through AMPA ion channels (which would be expected to promote excitotoxicity as discussed above) these agents are thought to mediate neuroprotection by increasing BDNF production (O'Neill *et al.*, 2004; O'Neill *et al.*, 2005). Consequently, AMPA positive allosteric modulators maintain to be evaluated for the treatment of PD in humans.

#### *1.5.2.1.2: NMDA receptors: Signal transduction, general distribution and therapeutic potential in PD*

NMDA receptors are composed of a family of seven subunits, NR1, NR2A-D and NR3A/B forming a tetrameric structure. Functional receptors contain NR1 together with one other NR2 subunit, or NR1 and both NR2 and NR3 subunits (Monyer *et al.*, 1992). The agonist binding site for glutamate is on the NR2 subunit and a co-agonist binding site for glycine exists on the NR1 subunits with receptor activation requiring occupancy of both sites (Kuryatov *et al.*, 1994). In the majority of cases, glutamate-mediated excitatory postsynaptic potentials (EPSPs) are comprised of a fast AMPA receptor mediated component that depolarises the cell and then a slower NMDA receptor mediated component. This is due to tonic inhibition of NMDA receptors by the  $Mg^{2+}$  present in the extracellular fluid. Thus if a sufficiently large enough depolarisation occurs, the  $Mg^{2+}$  block is relieved enabling opening of the NMDA channel permitting  $Ca^{2+}$  entry. It is this mechanism that underlies learning and memory, through a process of long term potentiation (LTP) (Lynch, 2004). As NMDA receptors desensitise slowly, the continuing rise in intracellular  $Ca^{2+}$  may also lead to excitotoxicity and consequent apoptosis.

NMDA receptors demonstrate expression in numerous regions of the basal ganglia, particularly the NR1 and NR2D subunits (Hallett & Standaert, 2004). NR2A and NR2B subunits are exclusively expressed in the striatum, where as NR2C subunits have only low expression in the SNpc (Hallett & Standaert, 2004). Therefore owing to their expression in the basal ganglia, coupled with their roles in synaptic



transmission and excitotoxicity, NMDA receptors have also been evaluated as therapeutic targets in PD.

Interestingly, an assortment of competitive antagonists (e.g. SDZ 220-581), non-competitive antagonists (e.g. MK-801 and dextrorphan), and glycine site antagonists (e.g. MRZ 2/570) have all been shown to reverse catalepsy and muscle rigidity induced by dopamine receptor blockade in rat models of PD (reviewed by Johnson *et al.*, 2009). MK-801 has also shown antiparkinsonian effects alone in 6-OHDA-lesioned rats as well as potentiating the effects of L-DOPA in both primate and rodent models of PD (Greenamyre *et al.*, 1994). Additionally MK-801 given by intra-subthalamic injection was shown to reduce mean firing rates of STN neurones by up to 20% which may underlie its antiparkinsonian efficacy (Allers *et al.*, 2005).

NMDA receptor antagonists such as MK-801 however, have been associated with severe side effects in larger mammals. Systemic administration of MK-801 has been reported to cause sedation in parkinsonian macaques (Crossman *et al.*, 1989), whilst ketamine, a NMDA antagonist used as a dissociative anaesthetic in humans can promote psychotomimetic action, memory deficits and addiction in low doses (Hallett & Standaert, 2004). Therefore, these complications have led to the search of more subtype-selective NMDA antagonists that might provide antiparkinsonian effects without cognitive side effects. One such target that has emerged from this search is the NR2B subunit which is almost exclusively expressed in the striatum. Indeed systemic administration of the NR2B selective antagonist ifenprodil relieves akinesia in reserpine-treated rats and MPTP-treated primates (Nash *et al.*, 1999; Nash *et al.*, 2000). In addition, it has been shown systemic oral treatment with a highly selective NR1A/NR2B subunit antagonist, CI-1041, can prevent the initiation of L-DOPA induced dyskinesias in MPTP-lesioned primates (Hadj *et al.*, 2004). In contrast, administration of an NR1/NR2B-selective NMDA antagonist, CP-101606 had no benefit on parkinsonian symptoms when administered as a monotherapy and provided only mild potentiation of L-DOPA-induced motor benefits (Nash *et al.*, 2004). Therefore NR2B subtype specific NMDA antagonists may offer an improved therapeutic alternative to the classical non-selective NMDA antagonists, although there is some disparity in efficacy between the different compounds tested.

#### *1.5.2.1.3: Kainate receptors: Signal transduction, general distribution and therapeutic potential in PD*

Kainate receptors comprise five subunits, separated into two groups; the KA1 and KA2 (which demonstrate higher affinity for kainate) and secondly GluR5-7 (Hollmann & Heinemann, 1994). Kainate receptors function as tetramers which likely exist in both homomeric and heteromeric configurations. Any combination of subunits is thought to be possible although only receptors formed from KA1 or KA2 subunits demonstrate a functional channel. Kainate receptors containing Glu5-6 subunits demonstrate a reduction in  $\text{Ca}^{2+}$  permeability (Burnashev *et al.*, 1995). The function of kainate receptors remain poorly understood, however it is thought their postsynaptic receptors may be involved in carrying part of the current charge required in signal transduction where a potential role in synaptic plasticity has been suggested (reviewed in Lerma, 2006). Kainate receptors, in contrast to AMPA and NMDA iGlu receptors reside both pre and post-synaptically, where they are thought to modulate neurotransmitter release (Rodriguez-Moreno *et al.*, 1997).

Kainate receptors are presumed to be widely distributed throughout the CNS, although there are currently few distribution studies to support this notion. Consequently, few studies have been performed to examine the antiparkinsonian potential of these receptors. However one study has shown that kainite GluR6/7 subunits expressed presynaptically on GABAergic pallidal terminals, function as heteroreceptors to modulate GABA release (Kane-Jackson & Smith, 2003). Furthermore, an electrophysiological study in the GP has shown kainate could significantly inhibit evoked inhibitory postsynaptic currents through a kainate mediated mechanism (Jin & Smith, 2007). This would suggest kainate receptors may have antiparkinsonian activity by decreasing the excessive GABA release within striatopallidal projections of the indirect pathway, although this notion remains to be determined.

### 1.5.2.2 Metabotropic glutamate receptors

Despite the promise of targeting iGlu receptors in the treatment of PD, their potential has been hampered by a lack of specificity in exclusively targeting overactive glutamatergic transmission. Owing to their wide distribution, blockade of these ion channel receptors can also interfere with learning and memory, potentially leading to severe side effects in humans. Thus focus has turned towards evaluating the metabotropic glutamate (mGlu) receptors as novel targets for the treatment of PD. In fact over the last few years, mGlu receptors have received a wealth of interest no doubt owing to their expression within the basal ganglia motor loop and ability to modulate glutamatergic transmission in a more subtle manner than iGlu receptors. Furthermore recent emphasis has focused more on developing ‘modulators’ rather than the classical ‘agonist’ which bind to a separate allosteric site and rely on the endogenous ligand i.e. glutamate to bind for activation. It is hoped this offers a far more exclusive approach to specifically targeting hyperactive glutamatergic transmission indicative of a diseased state as in PD. Since the focus of this thesis is to assess the therapeutic potential of targeting metabotropic glutamate receptors (particularly group III receptors) for PD, this family of receptors is discussed in greater detail below.

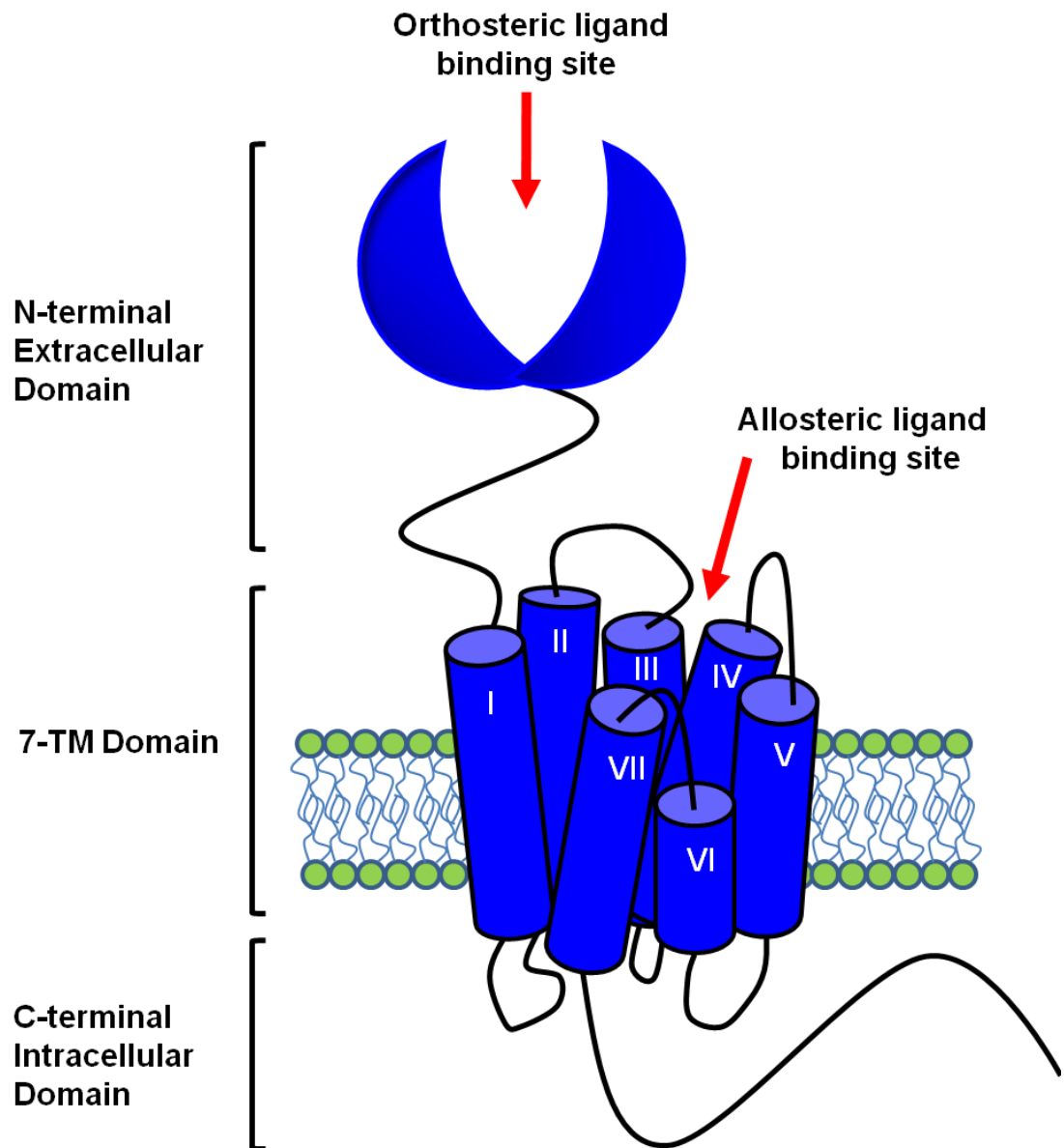
#### *1.5.2.2.1: Structure of metabotropic glutamate receptors*

The mGlu receptors are G-protein coupled receptors (GPCRs), which on the basis of sequence homolog belong to family C GPCRs along with the GABA<sub>B</sub> receptors, Ca<sup>2+</sup> sensing receptors, pheromone receptors, olfactory and taste receptors (Schoepp, 2001). As with many GPCRs, the mGlu receptors comprise a single polypeptide chain spanning seven transmembrane domains, where hydrophilic segments form the intracellular C-terminal domain and a characteristically large extracellular N-terminal domain. The N-terminal chain, forming two globular domains joined by a hinge region, similar to that of a Venus fly-trap configuration creates the ligand binding site for mGlu receptors (shown in figure 1.4; Conn & Pin, 1997). Interaction with the trimeric G-protein occurs in the central portion of the second intracellular loop, which is responsible for the selective recognition of the C-terminal end of the G-protein  $\alpha$ -

subunit (Havlickova *et al.*, 2003). The G-protein becomes activated following ligand binding which facilitates the exchange of GDP for GTP within the  $\alpha$ -subunit from the  $\beta\gamma$  subunits which are then able to regulate numerous enzymes and ion channels via secondary messengers to initiate numerous downstream cellular effects.

The mGlu receptors are sub-divided into three groups, Group I, II and III based on sequence homology, second messenger coupling and pharmacological profile. Group I mGlu receptors comprise mGlu1 and 5, Group II- mGlu2 and 3, and Group III – mGlu4, 6, 7 and 8 where further multiplicity is achieved by alternative splicing of group I and III (detailed in table 1.2). Functionality in this family relies on the formation of homodimers linked by disulphide bonds. The group I mGlu receptors can also form a heterodimer with  $\text{Ca}^{2+}$ -sensing receptors and adenosine  $\text{A}_1$  receptors, although such dimerisation has not been demonstrated for group II and III mGlu receptors (Jingami *et al.*, 2003). Glutamate is the predominant endogenous ligand at these receptors, however there is evidence L-cysteic acid and L-cysteine sulphinic acid can also activate group I mGlu receptors (Croucher *et al.*, 2001). *In situ* hybridisation and immunohistochemical studies reveal all mGlu receptors are found in abundance in the basal ganglia apart from mGluR6 which is exclusively expressed in the optic nerve.

Figure 1.4: Schematic illustration of a typical metabotropic glutamate receptor.



**Figure 1.4.** Schematic illustration of a metabotropic glutamate receptor with a characteristically large N-terminal extracellular domain that contains the orthosteric binding site for the endogenous ligand glutamate. The seven transmembrane domains (labelled in white) are connected by three intracellular and extracellular loops followed by a C-terminal tail. Allosteric ligands bind to an additional site to the orthosteric glutamate-binding site within the seven transmembrane domains. Abbreviations: 7-TM Domain: 7-transmembrane domain.

#### 1.5.2.2.2 Group I mGlu receptors: Signal transduction, general distribution and therapeutic potential in PD

The group I mGlu receptors (mGlu1a-d and 5a/b) are predominantly postsynaptic although have been located at pre-synaptic terminals. All members of the group I mGlu receptors couple to the  $G_q$  G-protein which is positively coupled to phospholipase C (PLC), hydrolysing membrane phosphoinositide to form inositol triphosphate ( $IP_3$ ) and diacylglycerol (DAG).  $IP_3$  induces  $Ca^{2+}$  release from  $IP_3$ -sensitive intracellular stores whilst DAG triggers protein kinase C (PKC) activity (Takechi *et al.*, 1998). Activation of group I mGlu receptors can also increase intracellular calcium in an  $IP_3$ -independent manner through opening of L-type voltage dependent  $Ca^{2+}$  channels and inhibition of  $K^+$  channels. In addition, the long intracellular C-terminal tail of mGlu1a and mGlu5a/b enables binding to Homer adaptor proteins (Tu *et al.*, 1998). Homer protein complexes allow mGlu receptors to come into close proximity with downstream targets. For example, homer connects mGlu1a and mGlu5a/b to ryanodine and  $IP_3$  receptors providing optimal organisation for  $Ca^{2+}$  release following receptor activation. Homer proteins also enable interaction between group I mGlu receptors and iGlu receptors, AMPA and NMDA (Tu *et al.*, 1999) which can lead to potentiation of excitatory responses due to increased intracellular  $Ca^{2+}$  concentration.

Group I mGlu receptors are widely expressed throughout the basal ganglia, preferentially within postsynaptic regions of the striatum, globus pallidus, STN and substantia nigra (Testa *et al.*, 1998). A degree of subtype differentiation exists since mGlu1 is found in abundance in the SNpc, where as mGlu5 is expressed predominantly in the striatum, STN and PFC with lower expression in other regions of the basal ganglia (Testa *et al.*, 1998; Messenger *et al.*, 2002). In the striatum, group I mGlu receptors are found both presynaptically on dopamine terminals that converge with MSNs and postsynaptically within the perisynaptic space on MSNs and interneurons (Gubellini *et al.*, 2004; Smith *et al.*, 2000; Zhang & Sulzer, 2003). In a state of elevated glutamate release, presynaptic group I mGlu receptors inhibit dopamine release by raising intracellular levels of  $Ca^{2+}$  triggering opening of  $Ca^{2+}$  sensitive  $K^+$  channels leading to hyperpolarisation (Zhang & Sulzer, 2003). However,

activation of postsynaptic mGlu5 receptors on MSNs potentiates NMDA currents (Pisani *et al.*, 2001). The activation of NMDA receptors can also potentiate mGlu5 receptor signalling by the activation of the protein phosphatase calcineurin, thus limiting receptor desensitisation through subsequent dephosphorylation of mGlu5 (Alagarsamy *et al.*, 1999).

The differential role of group I mGlu receptors within the striatofugal system is worthy of discussion here. With respect to the direct pathway, activation of group I mGlu receptors can inhibit D<sub>1</sub> mediated responses by modulating the activity of dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32). D<sub>1</sub> responses are normally amplified by the phosphorylation of DARPP-32 by protein kinase A (PKA), consequently converting DARPP-32 to an inhibitor of protein phosphatase 1. However, this pathway is inhibited following activation of group I mGlu receptors, by the stimulation of casein kinase 1 (CK1) and cyclin-dependent kinase 5 (CDK5), which phosphorylate DARPP-32, converting the protein to an inhibitor of PKA (Liu *et al.*, 2001). Thus, group I mGlu receptor activation inhibits the activity of D<sub>1</sub> projections of the direct striatofugal pathway.

In the indirect pathway, activation of group I mGlu receptors can suppress the actions of dopamine on MSNs that project to the GPe to increase neuronal excitability in the STN and SNpc. Furthermore, NMDA receptor currents in MSNs that project to the GPe are potentiated by ACh released by large spiny striatal interneurons in response to group I mGlu receptor activation (Gubellini *et al.*, 2004). Therefore, these findings suggest that blockade of group I mGlu receptors could be therapeutic by suppressing hyperactive glutamatergic transmission in overactive corticostriatal, subthalamonigral and subthalamopallidal pathways or cholinergic transmission in striatal interneurons. Since group I mGlu receptor activation opposes the action of dopamine, group I mGlu receptor antagonists have been evaluated for antiparkinsonian effects. Currently, focus has been on mGlu5 where chronic treatment with a selective mGlu5 negative allosteric modulator 2-methyl-6-(phenylethynyl)-pyridine (MPEP), significantly reversed akinesia in a bilateral 6-OHDA rodent model of PD (Breysse *et al.*, 2002). In addition, microinjection of MPEP into the STN of unilaterally 6-OHDA-lesioned rats significantly attenuated motor asymmetries with respect to

vehicle-treated animals, although microinjection into the SNpr and EP had no effect (Phillips *et al.*, 2006). These findings would suggest antiparkinsonian effects of MPEP are predominantly mediated via the STN which is supported by an electrophysiological study confirming mGlu5 modulates glutamatergic transmission in the STN. Furthermore, acute and sub-chronic intranigral administration of MPEP and LY367385 (mGlu1 antagonists) in 6-OHDA lesioned rats has been shown to significantly protect TH-positive cells in the SNpc with respect to vehicle-treated animals (Vernon *et al.*, 2007a). This is consistent with the notion that excitation of nigral dopaminergic neurones is regulated by the activation of group I mGlu receptors which may ultimately lead to excitotoxic effects.

To date, the antiparkinsonian efficacy of group I mGlu receptor antagonists has not been tested clinically thus only time will tell if transfer of these compounds to clinical trials will be deemed worthwhile. However, the anxiolytic, fenobam, discovered to be a negative allosteric modulator of mGlu5 receptor (Porter *et al.*, 2005), has previously been tested in Phase II clinical trials showing good anxiolytic efficacy and being generally well-tolerated, although some psychotomimetic activity was reported (Pecknold *et al.*, 1982). Therefore these previous clinical trials with fenobam may help to accelerate the transfer of group I mGlu antagonists into clinical trials for PD, should this be deemed to be worthwhile.

#### *1.5.2.2.3 Group II mGlu receptors: Signal transduction, general distribution and therapeutic potential in PD*

Group II mGlu receptors (mGlu2 and 3) are predominantly found presynaptically where they are usually located in pre-terminal axons (Shigemoto *et al.*, 1997). They function largely in a very similar manner to group III mGlu receptors which are discussed in section 1.5.2.2.4, therefore their mechanism of action will be discussed in more detail there. In brief, group II mGlu receptors are negatively coupled to adenylate cyclase via G<sub>i</sub> or G<sub>o</sub>-proteins resulting in a decrease in intracellular cAMP and presynaptic membrane hyperpolarisation (Conn & Pin, 1997). In addition, the  $\beta\gamma$ -subunit of group II mGlu receptors can inhibit L, N and P/Q type voltage dependent calcium channels. It is thought through these mechanisms group II mGlu receptors



can function as hetero- and auto-receptors by decreasing neurotransmission throughout the CNS at inhibitory and excitatory synapses respectively (Manzoni *et al.*, 1997; Salt & Turner, 1998).

Group II mGlu receptors are widely distributed throughout the basal ganglia. For example mGlu3 has shown robust gene expression throughout the motor loop particularly within the striatum (Messenger *et al.*, 2002). Using *in situ* hybridisation, mGlu2 expression has been located within the premotor cortex, striatum and STN (Messenger *et al.*, 2002; Ohishi *et al.*, 1998). In addition, mGlu3 but not mGlu2 has been shown on glial cells, close to excitatory synapses (Testa *et al.*, 1994). Indeed activation of mGlu3 receptors on astrocytes have been shown to be neuroprotective against NMDA-induced toxicity, which is thought to be driven by an increase in transforming growth factor- $\beta$  (TGF $\beta$ ) release (Bruno *et al.*, 2001).

Activation of group II mGlu receptors has been shown to inhibit glutamate release from corticostriatal and corticosubthalamic terminals (Lovinger & McCool, 1995). In addition, group II mGlu receptor activation on large aspiny interneurons has been shown to inhibit ACh release (Pisani *et al.*, 2003). Electrophysiological studies have revealed group II mGlu receptors may also modulate dopamine release where their activation reduces transmission at excitatory synapses onto dopaminergic neurones (Wigmore & Lacey, 1998). However, a contrasting study has shown using microdialysis in the striatum and *in vitro* release from nigral slice preparations, group II mGlu receptor agonists can cause an increase in dopamine release (Campusano *et al.*, 2002). Thus, the role of group II mGlu receptors in the regulation of dopaminergic function is unclear, although increasing dopamine release may well aid in normalising function of the striatofugal system in parkinsonian conditions. An important function of group II mGlu receptors has been revealed at the subthalamonigral terminals where activation of mGlu2 and 3 receptors have been shown to inhibit excitatory glutamatergic transmission (Bradley *et al.*, 2000). Therefore, these actions may well cause antiparkinsonian effects by reducing overactive excitatory drive from the STN to the GPi and SNpr output nuclei to normalise thalamocortical feedback.

The potential of targeting group II mGlu receptors for treatment in PD has been addressed in several animal models of PD. Indeed, *in vivo* studies using the group II mGlu receptor agonist LY379268 have shown i.c.v. administration reduces reserpine-induced akinesia although no antiparkinsonian efficacy was observed following systemic administration in the 6-OHDA-lesioned or reserpine-treated rat models (Murray *et al.*, 2002). This may be due to dopamine-mediated alterations in function or poor bioavailability in the brain. It is thought there are two predominant mechanisms to mediate neuroprotection following group II mGlu receptor activation, either by prevention of glutamate-mediated excitotoxicity or through release of neurotrophic factors such as TGF- $\beta$  from glial cells. An interesting study by Picconi *et al.*, (2002) using LY379268 and an additional agonist DCG-IV found greater sensitivity in depressing excitatory transmission in the corticostriatal synapse of 6-OHDA lesioned rodents with respect to controls. This hyper-responsiveness suggests group II mGlu receptors may become over-expressed as a compensatory mechanism in PD.

Although the antiparkinsonian effects of group II mGlu receptor agonists has not been tested in humans, they have however been tested in phase II clinical trials for anxiety disorders. Although the group II mGlu selective agonist LY354740 showed no anxiolytic activity, it was well tolerated revealing minor side-effects limited to gastrointestinal complaints (Bergink & Westenberg, 2005). Group II agonists should therefore be able to progress into clinical trials in PD, should this be deemed worthwhile.

#### *1.5.2.2.4 Group III mGlu receptors: Signal transduction*

The group III mGlu receptors (mGlu4a/b, 6, 7a/b and 8a/b) similar to group II are expressed predominantly presynaptically usually within the presynaptic zone (Shigemoto *et al.*, 1997). mGlu6 however is postsynaptic and is exclusively expressed in the retina, so will not be discussed further (Nakajima *et al.*, 1999). Group III mGlu receptors are coupled to  $G_i/G_o$  where their activation leads to inhibition of L,N, and P/Q type voltage dependent  $Ca^{2+}$  channels and activation of

presynaptic  $K^+$  channels (signalling for all the mGlu receptors are summarised in table 1.1).

Electrophysiological studies have confirmed both an auto and hetero-receptor role following activation of group III mGlu receptors serving to inhibit glutamate release on excitatory synapses (Turner & Salt, 1999) and GABA release on inhibitory synapses (Turner & Salt, 2003). As group III mGlu receptors have a relatively low affinity for glutamate and consequently are only activated by excess glutamate release, their function may be considered to be of feedback inhibition (Scanziani *et al.*, 1997). This property immediately raises the possibility that these receptors may play a key role in conditions where excitatory glutamate levels are raised, such as in PD. This potential is discussed at length in section 1.5.2.2.6.

The second messenger systems are not widely described for group III mGlu receptors although those involved in mGlu7 have been studied more extensively than other subtypes, therefore are discussed in more detail here as a likely example. Activation of mGlu7 is negatively coupled to adenylate cyclase via direct interaction with the G-protein, leading to a decrease in cAMP (Okamoto *et al.*, 1994). Following mGlu7 activation, fast inhibition of N-type  $Ca^{2+}$  channels occurs as a result of a direct interaction with the now dissociated G-protein  $\beta\gamma$  subunit and the  $\alpha_1$  subunit of the calcium channel (Waard *et al.*, 1997).

Group III mGlu receptors can also bind calmodulin (CaM) in the C-terminal by a  $Ca^{2+}$ -dependent process (Nakajima *et al.*, 1999). Indeed, numerous electrophysiological studies have demonstrated CaM binding can promote the dissociation of the G-protein  $\beta\gamma$  subunit (Dev *et al.*, 2001). Therefore, it has been suggested following nerve terminal depolarisation, high levels of intracellular  $Ca^{2+}$  allow group III mGlu receptors to mediate feedback inhibition of glutamate release via a  $Ca^{2+}$ /CaM-dependent mechanism. There is however an additional level of complexity within mGlu7 signalling since receptor activation is also positively coupled to PLC (Perroy *et al.*, 2000). This is somewhat of a surprise as this could lead to two mechanisms not indicative of an inhibitory role for mGlu7 receptors. The first of these is that PLC activation would cause an excitatory increase in intracellular

calcium. However this effect seems unlikely since this PLC mediated pathway has been shown to inhibit opening of P/Q type voltage-dependent  $\text{Ca}^{2+}$  channels (Perroy *et al.*, 2000). Secondly, PLC activation is known to activate PKC, a known inhibitor of CaM-mGlu7 binding. Indeed, phosphorylation at Ser862 within the mGlu7 C-terminus by PKC, inhibits CaM binding (Airas *et al.*, 2001) and thus negatively regulates mGlu7 activity. Therefore, the role of PLC/PKC-mediated pathways implicated in downstream signalling following mGlu7 activation may result in a form of negative feedback upon receptor activation, although the exact function of this mechanism remains to be determined.

An additional level of complexity within group III mGlu receptors is that mGlu4a, 7a, and 8a can all be phosphorylated by PKA, which effectively serves to inhibit the function of these receptors (Cai *et al.*, 2001). It is widely accepted that group III mGlu receptor inhibition of cAMP results in decreased PKA activity, promoting CaM binding and inhibition of  $\text{Ca}^{2+}$  channels. However,  $G_s$ -coupled presynaptic receptors which activate PKA can negatively regulate the function of group III mGlu receptors. Similar crosstalk has been reported between the  $\beta$ -adrenergic receptor and group III mGlu receptors where the  $\beta$ -adrenergic agonist, isoproterenol has been shown to block inhibition of EPSPs mediated by L(+)-2-amino-4-phosphonobutyric acid (L-AP4) in hippocampal slices (Cai *et al.*, 2001).

All group III mGlu receptor subtypes have been shown to interact with the adaptor protein PICK1 via a PDZ-binding domain in their C-terminal (El *et al.*, 2000). PICK1 has been shown to attenuate PKC $\alpha$ -evoked phosphorylation of mGlu7a to allow dissociation of  $\beta\gamma$  (Dev *et al.*, 2001). Although the exact interplay between PICK1, PKC and group III mGlu receptors is far from understood, the presence of PICK1 appears to be an absolute requirement for the inhibition of P/Q-type calcium channels by mGlu7a. Further proteins interacting with the group III mGlu receptor C-terminal domain include adaptor proteins, syntenin and GRIP, as well as protein phosphatase-1, which could be important in dephosphorylating Ser862 (Enz & Croci, 2003; Hirbec *et al.*, 2002). An additional group III mGlu receptor interacting protein, Filamin-A (Enz & Croci, 2003), induces the polymerisation of actin filaments, providing a

physical link between mGlu receptors and the actin cytoskeleton which could be important in regulating the macromolecular structure at the synapse.

A further level of complexity regarding the signalling of group III mGlu receptors has been shown by L-AP4 activating MAPK and phosphatidylinositol-3-kinase (PI3K) pathways, which may lead to a neuroprotective effect following activation of these receptors (Iacovelli *et al.*, 2002). L-AP4-mediated activation of group III mGlu receptors led to an activation of MAPK which was attenuated by overexpression of G-protein coupled receptor kinase-2 (GRK2) (Iacovelli *et al.*, 2004). The authors of this report suggest GRK2 may interact with mGlu4 via the  $\beta\gamma$  subunit, as this effect was not dependent on its kinase activity.

#### *1.5.2.2.5 Group III mGlu receptors: General distribution*

The mGlu4 receptor subtype has been found to be expressed throughout the CNS, where the most intense expression is in the cerebellar granule cells (Kinoshita *et al.*, 1996). High expression of mGlu4 is also found in the olfactory bulb, entorhinal cortex, hippocampus, lateral septum, amygdala, thalamic nuclei, lateral mammillary nucleus, pontine nuclei and dorsal horn (Ferraguti & Shigemoto, 2006). At the electron-microscope (EM) level, mGlu4 immunoreactivity has been localised to the presynaptic active zone, within the medial nucleus of the trapezoid body and the dentate gyrus (Elezgarai *et al.*, 1999; Shigemoto *et al.*, 1997).

The distribution of mGlu7a is the most extensively expressed of the group III mGlu receptors, where the most intense expression is present in the olfactory bulb, neurones of the medial septal nucleus and locus coeruleus (Kinoshita *et al.*, 1996; Shigemoto *et al.*, 1997). There is also high expression of mGlu7a in the neocortex, hippocampus, septum, claustrum, amygdala, hypothalamus, thalamus, SC, locus coeruleus and dorsal horn of the spinal cord (Ferraguti & Shigemoto, 2006). mGlu7b however, has a more limited expression and is usually found co-localised with mGlu7a (albeit to a lower level) in the neocortex, anterior thalamus, medial geniculate nucleus and locus coeruleus (Ferraguti & Shigemoto, 2006). At the EM level, both mGlu7a and mGlu7b

have been observed almost exclusively in the presynaptic active zone in axon terminals (Shigemoto *et al.*, 1997).

A more restricted pattern for mGlu8 is observed to that of mGlu7 (Corti *et al.*, 1998), although high mGlu8 expression has been seen in the olfactory bulb, hippocampus, lateral reticular nucleus of the medulla oblongata as well as the cerebral, piriform, and entorhinal cortices (Ferraguti & Shigemoto, 2006). The expression of mGlu8a is higher than mGlu8b whilst both generally overlap in terms of expression, although the spinal vestibular nucleus and lateral nucleus of the medulla oblongata, only express mGlu8a (Corti *et al.*, 1998). At the EM level mGlu8a has been observed in the presynaptic zone of axon terminals in the hippocampus (Shigemoto *et al.*, 1997).

#### *1.5.2.2.6 Group III mGlu receptors: Distribution and function in the basal ganglia*

All group III mGlu receptors are expressed within the basal ganglia aside from mGluR6. *In situ* hybridisation studies have shown high mRNA levels for mGluR4 and 7 within the striatum, moderate levels in the STN and lower levels in the SNpr, SNpc and GP (Messenger *et al.*, 2002). Moderate levels of mGluR8 mRNA were found in the striatum and STN although expression was low in all other structures of the basal ganglia (Messenger *et al.*, 2002). [<sup>3</sup>H]-L-AP4 binding studies in rat brain sections revealed high levels of binding in the GP and SNpr, with lower expression in the striatum, STN and SNpc (Hudtloff & Thomsen, 1998). These findings confirm receptor expression in the basal ganglia output structures, presumably on striatopallidal, striatonigral and subthalamonigral projections.

EM studies using antibodies directed against mGlu4 have revealed a presynaptic localisation of mGlu4 within the striatum, GPe, SNpr and EP (Corti *et al.*, 2002). In addition, mGlu4 immunoreactivity has been confirmed presynaptically at inhibitory synapses within the SNpr from subthalamonigral projections (Bradley *et al.*, 1999; Corti *et al.*, 2002). Immunoreactivity for mGlu7a has been shown within the striatum, GP and SNpr where EM studies have confirmed expression on axon terminals of corticostriatal and striatopallidal projections (Kosinski *et al.*, 1999). mGlu7a was also confirmed on corticostriatal terminals although this study did not investigate synapses

within the SNpr. Thus, mGlu7a expression on subthalamonigral terminals remains unproven, although mRNA expression within the STN would suggest mGlu7a is likely to be present at these terminals. Currently, no immunohistochemical studies have investigated the distribution of mGlu8 within the basal ganglia.

The widespread expression of group III mGlu receptors within the basal ganglia, coupled with their ability to modulate both excitatory and inhibitory transmission has rendered this group of mGlu receptors appealing targets for therapeutic modulation in PD. Electrophysiological studies have posed numerous beneficial effects group III mGlu receptor activation could provide in a parkinsonian basal ganglia. For example, at the striatopallidal synapse, activation of group III mGlu receptors was able to inhibit GABA<sub>A</sub>-mediated IPSPs through a presynaptic mechanism (Valenti *et al.*, 2003). Additionally these effects were absent in mGlu4 knock-out mice confirming an mGlu4-mediated response. As this is the first synapse in the indirect pathway which is thought to be heavily regulated by striatal D<sub>2</sub> receptors, mGlu4 receptor activation may be able to exert a dopamine-like effect by reducing transmission at this synapse. Indeed the benefits of targeting the GPe have been confirmed *in vivo*, where intrapallidal injections of the group III broad spectrum agonist, O-Phospho-L-serine (L-SOP) reversed reserpine-induced akinesia in rats (MacInnes *et al.*, 2004). Furthermore, i.c.v. administration of the positive allosteric modulator of mGlu4, N-Phenyl-7-(hydroxyamino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), demonstrated activation of the mGlu4 receptor alone can reverse reserpine-induced akinesia in rats (Marino *et al.*, 2003). However the effects of PHCCC in this study could also be due to modulation of glutamate release further downstream at the subthalamonigral synapse.

Electrophysiological studies within the SNpr have demonstrated activation of group III mGlu receptors by L-AP4 was able to inhibit glutamate-mediated EPSPs, via a presynaptic mechanism (Wittmann *et al.*, 2001). Furthermore, L-AP4 also inhibits excitatory neurotransmission in the SNpc, an effect potentiated by the mGlu4 positive allosteric modulator (PAM) PHCCC, suggesting mGlu4 receptor activation may be key to this response (Valenti *et al.*, 2005). Therefore, the potential benefits of group III mGlu receptor activation in the parkinsonian substantia nigra are two-fold; (i) to

decrease excess glutamate release in the SNpr, therefore normalising thalamocortical feedback and potentially reversing akinesia; and (ii) to decrease excess glutamate release in the SNpc, which may be responsible for neurodegeneration of dopaminergic neurones by excitotoxicity. It is the potential of group III mGlu receptor activation to mediate neuroprotection that the studies conducted in this thesis set out to address. A recent report demonstrating that sub-chronic intranigral administration of L-AP4 provided neuroprotection of nigral TH positive cells in the 6-OHDA model of PD provided the initial support to this aim (Vernon *et al.*, 2007b).

Group Number	Receptor subtypes (splice variants)	G-protein	Effects of activation
1	mGlu1 (a,b,c,d) mGlu5 (a,b)	G <sub>q/o</sub> G <sub>q</sub>	↑ [Ca <sup>2+</sup> ] ↑ DAG, IP <sub>3</sub> ↑ L-VDCC
2	mGlu2 mGlu3	G <sub>i/o</sub>	↓ [Ca <sup>2+</sup> ] ↓ cAMP ↓ VDCC
3	mGlu4 (a,b) mGlu6 mGlu7 (a,b) mGlu8 (a,b)	G <sub>i/o</sub>	↓ [Ca <sup>2+</sup> ] ↓ cAMP ↓ VDCC

**Table 1.2:** Summary of the classification and signalling of the different mGlu receptor subtypes. Abbreviations: DAG: Diacylglycerol; IP<sub>3</sub>: Inositol-1,4,5-phosphate; cAMP: cyclic adenosine monophosphate; L-VDCC: L-type voltage-dependent calcium channels VDCC: voltage-dependent calcium channels; ↑: Increase or activation; ↓: decrease of inhibition.



## 1.6 General hypothesis

Activation of  $G_{i/o}$  coupled group III mGlu receptors leads to inhibition of pre-synaptic  $Ca^{2+}$  channels and hence transmitter release from neurons in which they reside. Group III mGlu receptors, notably mGlu4,7 and 8 are believed to be located pre-synaptically on subthalamonigral neurons.

We hypothesise activation of group III mGlu receptors on the subthalamonigral pathway by inhibiting glutamate transmission in the SNpc may provide functional neuroprotection in a rodent model of PD.

## **1.7 Broad aims of this thesis**

In order to investigate our hypothesis, the overall aim of the studies described in this thesis was to delineate the potential of targeting group III mGlu receptor subtypes to provide functional neuroprotection in the 6-OHDA rodent model of PD.

In Chapter 2 of this thesis, preliminary immunohistochemical studies were undertaken to investigate the distribution patterns of group III mGlu receptors within the basal ganglia motor loop, with special focus on their distribution within the SNpc.

In Chapter 3, an initial study was conducted to confirm the ability of broad spectrum agonist-induced activation of group III mGlu receptors to provide neuroprotection in the 6-OHDA lesion rat model of PD. These studies expanded on existing knowledge to assess if protection at the post-mortem level translated to preservation in motor function. Next, the pharmacological identity of the group III mGlu receptor mediating this protective effect was examined. Previous reports in the literature had suggested the mGlu4 receptor may be a key subtype in mediating neuroprotective effects therefore focus of Chapter 4 was to assess the potential of targeting mGlu4 receptors to provide functional neuroprotection in the 6-OHDA rat model of PD.

Further studies were performed using a similar experimental paradigm to assess the potential of targeting mGlu7 and mGlu8 receptor subtypes, the findings of which are discussed in Chapter 5. Within Chapters 4 and 5, attempts were also made to begin to elucidate mechanisms involved in mediating neuroprotective effects. Previous work in this lab had already implicated inhibition of glutamate release in the actions of these agents; here potential neuroinflammatory mechanisms were explored using immunocytochemistry to assess astroglial and microglial function following treatment with selective group III mGlu receptor agents. By conducting these investigations it was hoped that the data generated would provide evidence in support of group III mGlu receptors subtypes as an alternative, non-dopaminergic, strategy to provide much needed disease modification in the treatment of PD.

**Chapter 2:** Preliminary investigation to assess the distribution of group III metabotropic glutamate receptors in the basal ganglia of naïve rat brain

## 2.1 Introduction

Before we set out to investigate the neuroprotective potential of targeting group III mGlu receptors using selective pharmacological ligands, this preliminary study sought to determine the expression profile of each of the subtypes throughout the basal ganglia. As previously mentioned, there are four group III mGlu receptor subtypes, mGlu4, 6, 7 and 8; all of which are widely expressed throughout the CNS, with the exception of mGlu6 which is largely restricted to the retina (discussed in Chapter I, section 1.5.2.2.5). These G<sub>i</sub>/G<sub>o</sub>-coupled receptors are found predominantly on pre-synaptic terminals of GABAergic and glutamatergic neurones where they are involved in regulating synaptic transmission, most likely through inhibition of voltage-gated calcium entry required for triggering transmitter release (Conn & Pin, 1997; Trombley & Westbrook, 1992). These receptors have also been demonstrated at postsynaptic sites in some brain regions where their signalling through activation of G-protein coupled inwardly rectifying potassium channels is expected to produce membrane hyperpolarisation (Saugstad *et al.*, 1996).

Localization of group III mGlu receptors within the basal ganglia has been unravelled to some extent through a combination of in situ hybridization, immunohistochemistry and electron microscopy studies in rodents. Indeed, our understanding of the distribution of mGlu4 and mGlu7 receptors has not changed significantly in the last decade (compare Rouse *et al.*, 2000 with Duty, 2010). Both mGlu4 and mGlu7 receptors exhibit a predominantly pre-synaptic distribution. They have been found on terminals of the glutamatergic corticostriatal pathway which is considered to be overactive in PD and thereby contribute to the increased firing of the indirect basal ganglia pathway (Obeso *et al.*, 2008). Furthermore, autoradiographic visualisation of group III mGlu receptors, using the broad spectrum group III mGlu agonist [<sup>3</sup>H]-L-AP4, has shown high levels of binding within the GP and SNpr, moderate levels in the striatum, and low levels within the STN and SNpc in rat brain (Hudtloff & Thomsen, 1998). In support of this finding, mGlu4 and mGlu7 receptors have been found on terminals of the GABAergic striatopallidal and striatonigral pathways (Bradley *et al.*, 1999; Corti *et al.*, 2002; Kosinski *et al.*, 1999) as well as on excitatory (presumed glutamatergic) terminals in the SNr (Kosinski *et al.*, 1999; Corti *et al.*,

2002). While the SNr receives excitatory inputs not only from the STN but also from the pedunclopontine nucleus and frontal cortex (Carter, 1982; Di Loreto *et al.*, 1992; Kita & Kitai, 1987), the presence of mGlu4 and mGlu7 mRNA in the STN (Kosinski *et al.*, 1999; Messenger *et al.*, 2002; Testa *et al.*, 1994) strongly supports their expression on terminals of STN efferents in the so-called subthalamonigral pathway. In a study in which antibodies were raised against the mGlu4 receptor, specifically the mGlu4a splice variant which demonstrates similar distribution patterns to the mGlu4b variant, showed co-localisation with the pre-synaptic terminal marker, synaptic vesicle protein-2, suggesting these receptors are located pre-synaptically on afferents in the SNpr (Bradley *et al.*, 1999; Iversen *et al.*, 1994). Whether these were subthalamonigral or striatonigral terminals was not examined.

The distribution of mGlu8 receptors in the basal ganglia is the least well characterized of all, although expression of mRNA encoding mGlu8 receptors has been reported in the cortex, striatum and STN (Messenger *et al.*, 2002). Therefore, it remains a possibility that mGlu8 receptors are found on pre-synaptic terminals of corticostriatal, striatonigral and subthalamonigral pathways. In addition, binding studies comparing [<sup>3</sup>H]-L-AP4 (30nM) binding in wild-type and mGlu4 receptor knock-out mice revealed 28% of specific binding remains in the SNpr of the knock-out mice (Thomsen & Hampson, 1999). Although these results confirm a significant proportion of L-AP4 binding is due to mGlu4 in this region, the remaining high-affinity specific binding could be due to mGlu8. This study used a concentration of 30 nM L-AP4, therefore the binding sites observed most likely reflect mGlu4 and mGlu8 binding, rather than mGlu7 which requires higher ( $\mu$ M range) concentrations for affinity (Cartmell & Schoepp, 2000). Unfortunately, no mGlu8 knock-out studies have been used to confirm this supposition. Therefore, the presence of mGlu8 in the SN remains uncertain.

Taken together, these findings described above confirm group III mGlu receptors are widely distributed throughout the basal ganglia although some uncertainties remain. Studies investigating mRNA expression patterns, by themselves, are insufficient for the quantitative description of biological systems since they fail to account for post-transcriptional mechanisms controlling the protein translation rate (Gygi *et al.*, 1999),

the half-lives of specific proteins or mRNAs (Varshavsky, 1996) and the intracellular location and molecular association of the protein products (Urlinger *et al.*, 1997). For example, in some genes expressed in the yeast *Saccharomyces cerevisiae*, while the mRNA levels were of the same value, the protein levels varied by more than 20-fold (Gygi *et al.*, 1998). With this in mind, one must be cautious in assuming the presence of group III mGlu receptor mRNA is indicative of protein expression. Furthermore, it can be difficult to construct valid comparisons between group III mGlu receptor subtype expression from separate immunohistochemical studies, using different antibodies, likely of varying specificity and affinity, utilising various tissue processing methods. Indeed, generally when comparing the immunocytochemical results obtained from two laboratories, the results often differ (Hemmings *et al.*, 2009). In addition to this, mGlu8 receptor distribution in the basal ganglia still remains largely unexplored. Thus, the main objective of the studies outlined in this chapter was to provide a comprehensive analysis of group III mGlu receptor expression, examining each subtype in all regions of the basal ganglia motor loop. Of particular interest to these studies would be the presence of group III mGlu receptor expression in the SNpc. However, since in PD loss of dopaminergic neurones in this nucleus leads to degeneration in the terminal regions of the striatum and downstream changes in both the direct and indirect motor circuits, it was considered of interest to investigate receptor expression in each of the basal ganglia nuclei. The distribution of mGlu4 and mGlu7 has already been investigated in a number of basal ganglia nuclei however, a single study has yet to reveal a complete distribution profile.

## 2.2 Aims

The overall aim of this preliminary study was to investigate the expression profile for the group III mGlu receptor subtypes mGlu4, 7 and 8 throughout the basal ganglia. Group III mGlu receptor expression was assessed in the basal ganglia nuclei of the striatum, GPe, EP/GPi, STN and SNpc/SNpr in naïve rat brain.

More specifically the key objectives were:

1. to conduct immunohistochemical studies using antibodies raised against specific group III mGlu receptor subtypes to confirm the expression profiles of mGlu4 and 7 in the SNpc and additional basal ganglia nuclei of interest
2. to investigate the expression profile of mGlu8 in the SNpc and for completeness, all the major basal ganglia nuclei

## 2.3 Methods

### 2.3.1 General methodological considerations

#### 2.3.1.1 Animals

In studies of this nature it is important to consider the effect of age upon the parameters under investigation. Numerous changes in group I and group II mGlu receptor expression within ageing rats has been previously reported (Simonyi *et al.*, 2000), thus it is pertinent to suggest similar effects would likely occur to group III mGlu receptor expression. Indeed a significant reduction in mGlu4 mRNA in the cerebellar granule layer and mGlu7 in the cortex has been shown in aged rats (Simonyi *et al.*, 2000). The effects of ageing on mGlu8 expression remain to be determined. It has also been suggested mGlu receptor function may also be compromised in ageing rats, whereby mGlu5 mediated LTP responses were lost in older rats (Wang *et al.*, 2007b).

Female reproductive hormones have also been shown to have a profound effect on previous studies similar to those described in this thesis. For example, there are many examples where oestrogen, in particular oestradiol, has been shown to interfere with group I and group II mGlu receptor-mediated responses (Boulware *et al.*, 2005; Hilton *et al.*, 2006; Rao & Sikdar, 2006). Although to date there is no evidence oestrogen can affect group III mGlu receptor responses, this is a clear possibility owing to the high sequence homology shared with group I and II. There is also evidence to suggest differences in oestrogen levels during the reproductive cycle alters the susceptibility of nigral dopaminergic neurones to 6-OHDA. For example, loss of dopaminergic neurones in the substantia nigra was greater in animals lesioned at diestrus (low oestrogen), when compared with animals lesioned at proestrus (high oestrogen). Additionally, the density of the dopamine transporter (DAT), for which 6-OHDA toxicity is dependent, was higher at diestrus (Datla *et al.*, 2003). Furthermore, oestradiol has been implicated in preventing glutamate-mediated excitotoxicity through group I mGlu receptors (Hilton *et al.*, 2006). Collectively these studies highlight a neuroprotective role for oestrogen, rendering female rats unsuitable for



studies investigating neuroprotection in the 6-OHDA lesion model, as described in this thesis. Thus, to avoid any potential age or sex related alterations to group III mGlu receptor expression or function, male Sprague Dawley rats weighing between 270-300g, 8-10 weeks in age, were selected for all studies described in this thesis.

#### **2.3.1.2 Antibodies for group III mGlu receptor subtypes**

The group III mGlu receptor subtype antibodies selected for these studies were purchased from commercially available sources. Each of the primary antibodies used in these studies were synthetic peptides derived from the corresponding human equivalent raised in rabbit. With each of the group III mGlu receptor subtypes, western blot analysis conducted by the commercial supplier (Abcam), demonstrated positive immunoreactivity was blocked using an immunising peptide. Each of the antibodies used had previously been used in rat tissue, with the exception of mGlu7 which was predicted to react with rat based on the high sequence homology with human mGlu7 receptors (~99%).

### **2.3.2 Specific methodological details**

#### **2.3.2.1 Animals**

Male Sprague Dawley rats (B & K or Harlan, U.K.) weighing 270 – 300 g were used in these studies. Food and water were provided *ad libitum*. Animals were housed in a temperature- and humidity-controlled environment with a 12-h light / dark cycle. All procedures conformed to the U.K. Animals (Scientific Procedures) Act, 1986 and every effort was made to minimise animal numbers and suffering.

#### **2.3.2.2 Immunohistochemical protocol**

Two naïve rats were deeply anaesthetised using pentobarbital (100mg kg<sup>-1</sup>, i.p.) and transcardially perfused with 200ml 100mM phosphate-buffered 0.9% saline (PBS, composition in mM: NaCl 137, KCL 2.7, KH<sub>2</sub>PO<sub>4</sub> 1.8, Na<sub>2</sub>HPO<sub>4</sub> 10), followed by 200ml 4% paraformaldehyde (PFA) in PBS. Brains were quickly removed and placed

in fixative overnight to ensure complete fixation. The brain was then cut into a rostral (striatal) and caudal (SN) segment, dehydrated and defatted before being manually embedded into paraffin wax. Coronal sections (6µm) containing all basal ganglia regions of interest (striatum, GPe, GPi and SNpr/pc), according to the rat brain atlas (Paxinos & Watson., 1998) were cut on a microtome and then transferred into a water bath kept at ~45°C, in which they were left to float on the water surface for a few minutes until the sections had expanded fully. Sections were then collected onto Superfrost Plus glass microscope slides, firmly pushed down against the slide using blotting paper dampened with 30% industrial methylated spirit (IMS), and incubated overnight at 60°C to ensure adhesion to the slide. Schematic images of these sections are presented in figure 2.1.

For each animal, three adjacent sections from the region of interest were prepared for immunohistochemistry, firstly, by dewaxing and dehydrating them. Sections were immersed in 100% xylene for 2 x 5min time-periods and subsequently immersed in 100% IMS for 4 x 2min time-periods, with solution changes separating each immersion. Sections were then immersed for 10min in 3% hydrogen peroxide to block endogenous peroxide activity. Thereafter, antigenicity was restored in the tissue sections using citric acid antigen retrieval. This was done by boiling the dewaxed sections in citric acid (1M, dissolved in dH<sub>2</sub>O, pH 6.0) for 8min in a microwave pressure cooker. After this, sections were removed from the citric acid solution and thoroughly rinsed in dH<sub>2</sub>O to wash away any remaining buffer solution. Excess dH<sub>2</sub>O was removed from the slides/sections by dabbing them lightly on blotting paper, after which a PAP pen was used to apply a hydrophobic barrier around each brain section. Sections were then incubated in blocking buffer (1% BSA in 0.5M TBS and 10% sodium azide, pH 7.6) for 10 min to block non-specific binding sites present in the tissue sections. The blocking solution was then removed, and the sections incubated with the primary antibody (rabbit polyclonal anti mGlu4-8, Abcam; dilutions are shown in Table 2.1) overnight at room temperature (RT). After washing in 0.1M TBS for 10 min to remove any unbound antibody, sections were incubated with a polyclonal secondary antibody (goat anti-rabbit IgG-biotinylated 1:200, Sigma) for 2 hours at RT. An avidin-biotin-HRP complex was freshly prepared using an ABC kit (Vector Labs). This was done by mixing/diluting solution A (avidin) with solution B

(biotinylated HRP) in an appropriate ratio in TBS (10µl of solution A was added to every 1ml of 0.5M TBS, pH 7.6). Sections were washed for 10 min in TBS to remove any excess unbound secondary antibody and then incubated for 30 min in the freshly prepared avidin-biotin-HRP complex to allow the complex to conjugate to the biotinylated secondary antibody bound to the sections. Sections were then washed in TBS to remove any excess unbound avidin-biotin HRP complexes. Finally, antigen localisation was visualised by incubating sections in diaminobenzidine tetrahydrochloride (DAB) (0.05% DAB/0.03% H<sub>2</sub>O<sub>2</sub>) solution dissolved in TBS (0.1M TBS, pH 7.6) for 10 min. Following this, sections were removed from the DAB solution and thoroughly washed in dH<sub>2</sub>O to remove any remaining DAB solution from the slides. Sections were then dehydrated in 100% IMS for 4 x 2min time-periods and then subsequently cleared in 100% xylene for 2 x 5min time-periods, with solution changes separating each immersion period. Finally slides were cover-slipped with DPX mountant and allowed to dry overnight before being analysed using light microscopy. Digital images of the target nuclei from triplicate sections were captured using a Zeiss apotome microscope or an Epson Perfection V700 Photo scanner for qualitative visualisation of the relative levels of immunostaining, and recorded using Axiovision LE software (Carl Zeiss Ltd).

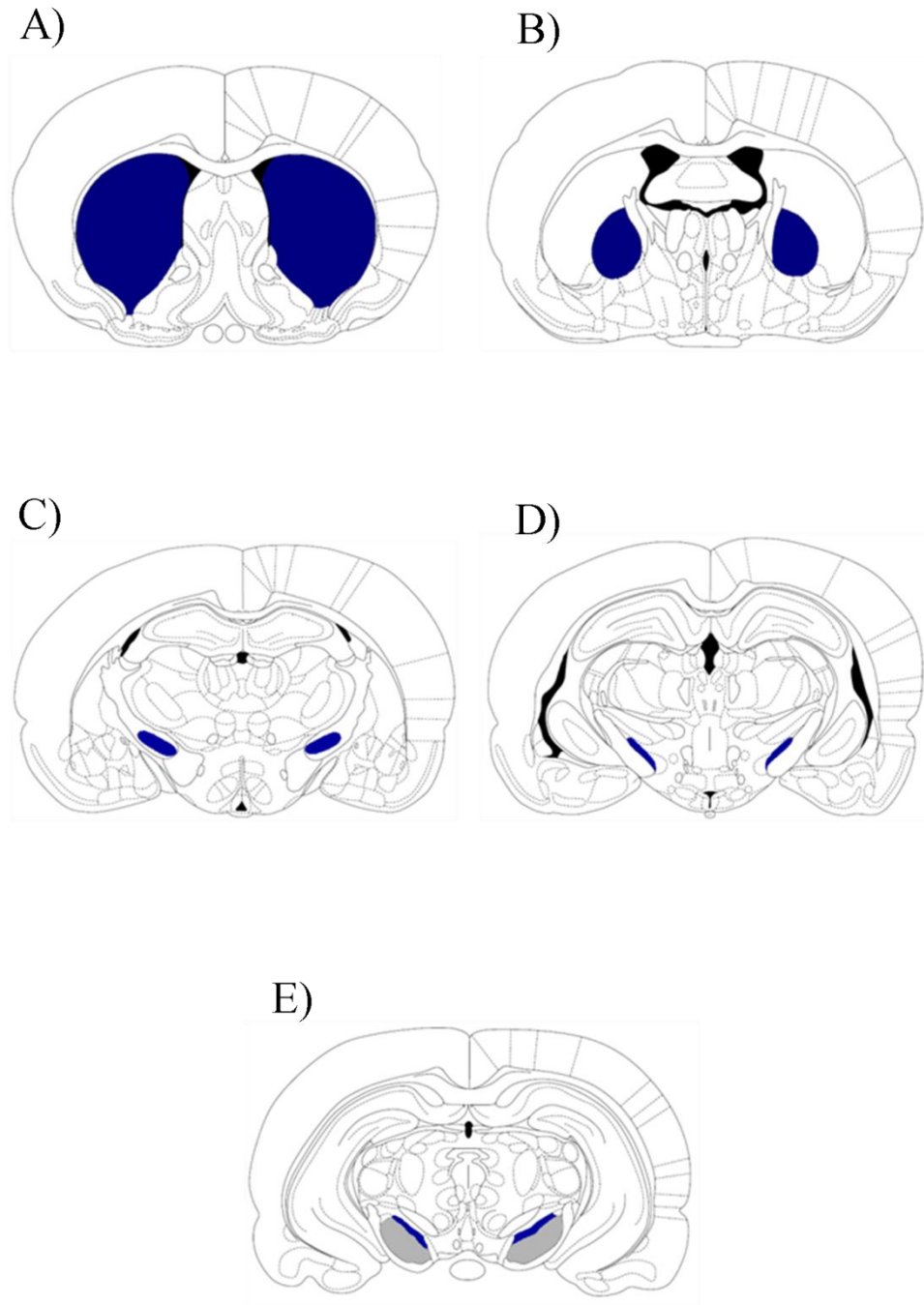
### 2.3.2.3 Materials and suppliers

For details of all reagents, consumables and supplier contacts refer to Appendix I.

Supplier	Primary antibody	Dilution
Abcam	Rabbit polyclonal anti-mGlu4 (ab53088)	1:100
Abcam	Rabbit polyclonal anti mGlu7 (ab53705)	1:200
Abcam	Rabbit polyclonal anti mGlu8 (ab53094)	1:50
	<b>Secondary antibody</b>	
Dako	Goat anti-rabbit IgG-biotinylated	1:200

**Table 2.1:** Primary and secondary antibodies used in immunohistochemical studies.

Figure 2.1: Anatomical positioning of the basal ganglia nuclei in the rodent brain.



**Figure 2.1:** Schematic of coronal sections from the rat brain atlas (Paxinos & Watson, 1998) highlighting the basal ganglia nuclei of interest in blue. **(A)** Striatum, +0.20 mm anterior posterior (AP) from bregma. **(B)** External globus pallidus (GPe), -0.92 mm AP from bregma. **(C)** Entopeduncular nucleus (EP) or Internal globus pallidus in primates and humans (GPi), -2.56 mm AP from bregma. **(D)** Subthalamic nucleus (STN), -4.16mm AP from bregma. **(E)** Substantia nigra pars compacta/ reticulata (grey) (SNpc/pr), -5.30 mm AP from bregma.

## **2.4 Results**

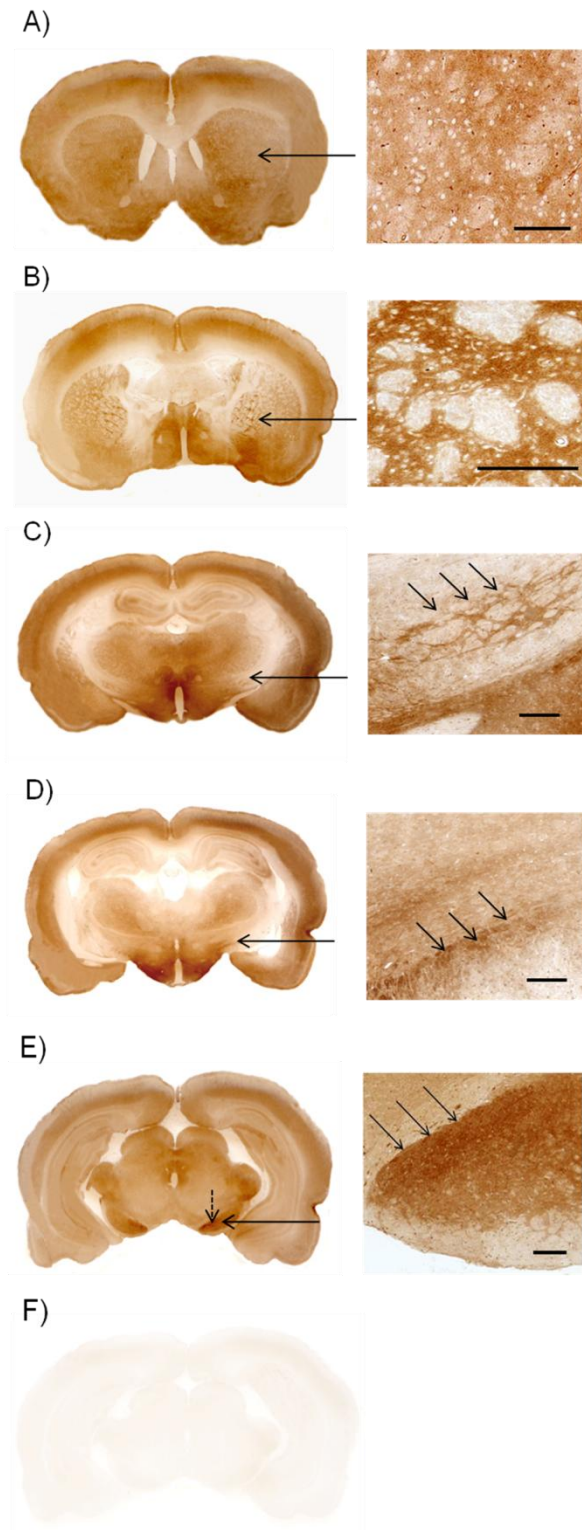
### **2.4.1 Distribution of group III mGlu receptor subtypes within the rodent basal ganglia**

#### **2.4.1.1 mGlu4 receptor**

The regional distribution of mGlu4 receptors was investigated in the rat basal ganglia by light microscopic immunohistochemistry using mGlu4 specific antibodies utilising the avidin-biotin-horseradish peroxidase complex to activate the diaminobenzidine (DAB) reagent. Immunoreactivity with the mGlu4 antibodies demonstrated widespread DAB labelling throughout the brain and by qualitative analysis revealed reproducible distribution patterns in both animals examined. The results from a representative set of sections are shown in figure 2.2.

Within the basal ganglia nuclei, immunoreactivity was most intense in the striatum (figure 2.2A), the external globus pallidus (GPe, figure 2.2B), SNpr (figure 2.2E), and of particular interest to these studies, the SNpc (figure 2.2E). Moderate immunoreactivity was also seen in the other basal ganglia nuclei, the entopeduncular nucleus (EP/internal globus pallidus, GPi; figure 2.2C) and the subthalamic nucleus (STN; figure 2.2D). On closer examination of magnified images from each of the target nuclei (shown in the right hand panels of figure 2.2), staining for mGlu4 appeared predominantly of a diffuse fibrous nature, suggesting a likely pre-synaptic, axon terminal location. The 'no primary antibody' control displayed negative DAB staining following application of only the anti-rabbit secondary antibody demonstrating no non-specific background staining of the secondary antibody (figure 2.2F). In summary, the qualitative order of expression for mGlu4 in the rat basal ganglia was SNpc > SNpr > GPe > Striatum > STN  $\geq$  GPi.

Figure 2.2: Distribution of mGlu4 immunoreactivity in the rat basal ganglia.



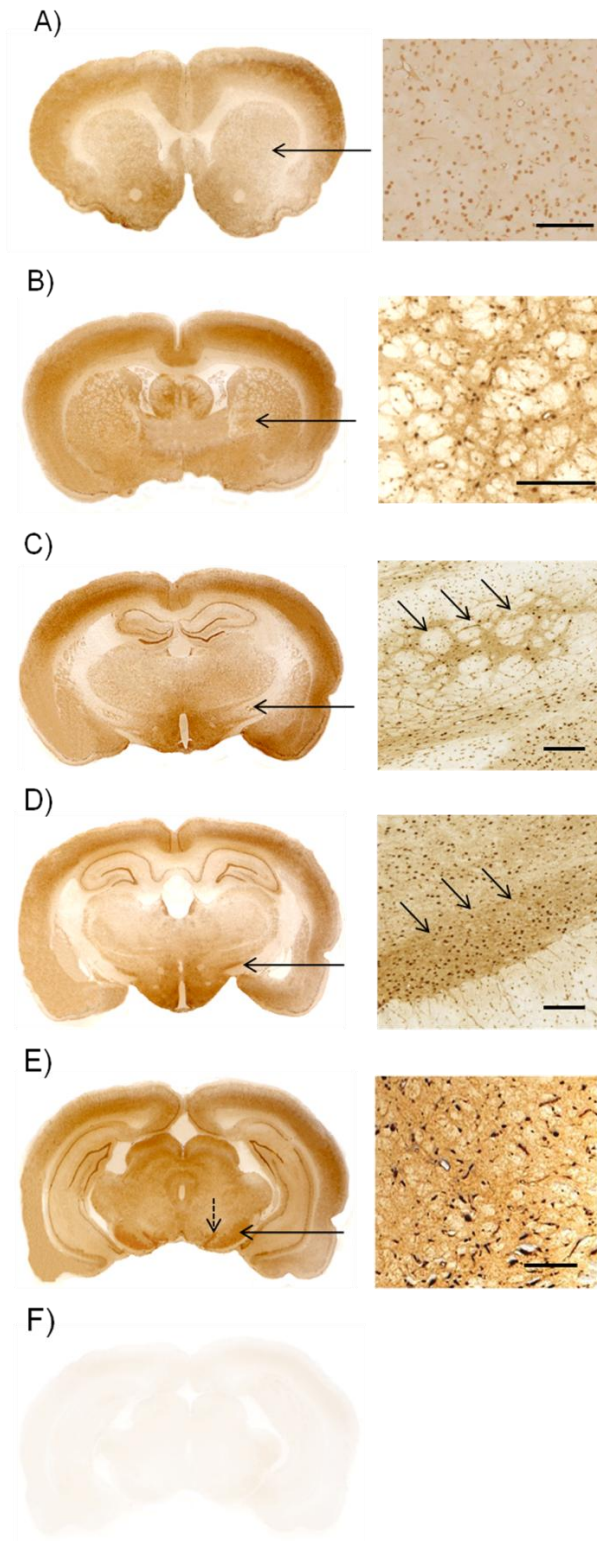
**Figure 2.2:** Photomicrographs showing the distribution of immunoreactivity for the mGlu4 receptor in the rodent basal ganglia. mGlu4 immunoreactivity is shown in (A) the striatum, (B) the external globus pallidus (GPe), (C) the entopeduncular nucleus (EP) or internal globus pallidus in primates and humans (GPi [arrow]), (D) the subthalamic nucleus (STN [arrow]) and (E) the substantia nigra pars compacta/ reticulata (SNpc [dashed arrow]/ SNpr [arrow]) and (F) no primary antibody control, showing immunoreactivity of the secondary anti-rabbit antibody in the substantia nigra. Arrows in the right hand panel indicate dorsal border of target nuclei. Scale bars equal 200µm.

#### **2.4.1.2 mGlu7 receptor**

The regional distribution of mGlu7 receptors was investigated in the rat basal ganglia by light microscopic immunohistochemistry using mGlu7 specific antibodies coupled with DAB staining, with the results shown in figure 2.3. Immunoreactivity with the mGlu7 antibodies showed widespread DAB labelling throughout the brain.

Within the basal ganglia nuclei, the most intense immunoreactivity was seen in the subthalamic nucleus (STN; figure 2.3D) and substantia nigra pars reticulata (SNpr; figure 2.3E). There was lower, but still moderate intensity, in the striatum (figure 2.3A), external globus pallidus (GPe; figure 2.3B) and the entopeduncular nucleus (EP/ internal globus pallidus; figure 2.3C). Of particular interest to these studies was the notably high mGlu7 immunoreactivity observed in the substantia nigra pars compacta, particularly within the medial segment of the dorsal tier (SNpc; figure 2.3E). Generally, staining for mGlu7 was diffuse and also punctate in nature suggesting pre but also post-synaptic origin. On closer inspection, mGlu7 staining in some circumstances demonstrated co-localisation with nuclei suggesting a degree of non-specific binding with this antibody (shown in the right hand panels of figure 2.3). The 'no primary antibody' control displayed negative DAB staining following application of only the anti-rabbit secondary antibody demonstrating no non-specific background staining of the secondary antibody (figure 2.3F). In summary, the qualitative order of expression for mGlu7 in the rat basal ganglia was  $STN \geq SNpr \geq SNpc > GPe \geq GPi \geq Striatum$ .

Figure 2.3: Distribution of mGlu7 immunoreactivity in the rat basal ganglia



**Figure 2.3:** Photomicrographs showing the distribution of immunoreactivity for the mGlu7 receptor in the rodent basal ganglia. mGlu7 immunoreactivity is shown in (A) the striatum, (B) the external globus pallidus (GPe), (C) the entopeduncular nucleus (EP) or internal globus pallidus in primates and humans (GPi [arrow]), (D) the subthalamic nucleus (STN [arrow]) and (E) the substantia nigra pars compacta/ reticulata (SNpc [dashed arrow]/ SNpr [arrow]) and (F) no primary antibody control, showing immunoreactivity of the secondary anti-rabbit antibody in the substantia nigra. Arrows in the right hand panel indicate dorsal border of target nuclei. Scale bars equal 200µm.

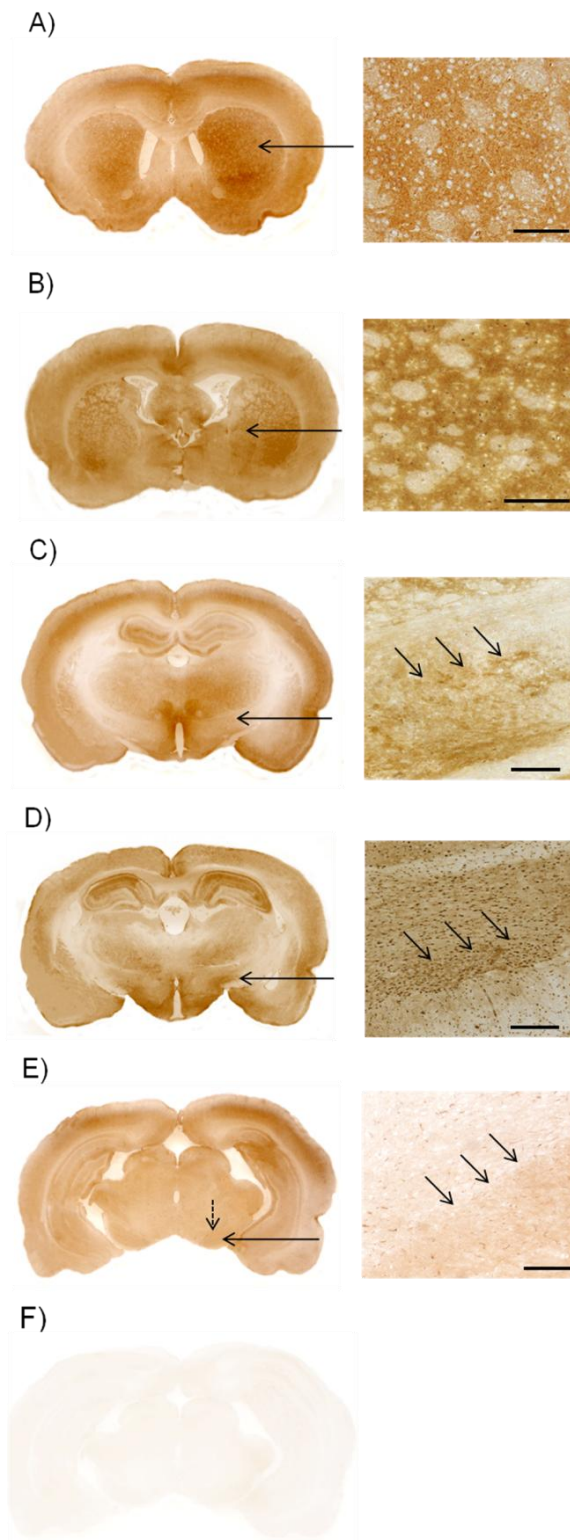


### 2.4.1.3 mGlu8 receptor

The regional distribution of mGlu8 receptors was investigated by light microscopic immunohistochemistry using mGlu8 specific antibodies coupled with DAB staining, with results shown in figure 2.4. Immunoreactivity with the mGlu8 antibodies demonstrated widespread DAB labelling throughout the brain, although at a much lower intensity to that seen with mGlu4 or 7.

Within the basal ganglia nuclei, the most intense immunoreactivity was seen in the striatum (figure 2.4A) and the external globus pallidus (GPe, figure 2.4B). There was also moderate immunoreactivity in the entopeduncular nucleus (EP/internal globus pallidus, GPi; figure 2.4C) and the subthalamic nucleus (STN; figure 2.4D) but low immunoreactivity in the substantia nigra pars reticulata (SNpr; figure 2.4E). Of particular interest to these studies was the very low intensity of mGlu8 immunoreactivity observed in the substantia nigra pars compacta (SNpc; figure 2.4E). Staining for mGlu8 in the SNpc revealed a similar distribution pattern to mGlu7 demonstrating particular, albeit low intensity, staining in the medial segment with little to no immunoreactivity in the lateral region. The 'no primary antibody' control displayed negative DAB staining following application of only the anti-rabbit secondary antibody demonstrating no non-specific background staining of the secondary antibody (figure 2.4F). In summary, the qualitative order of expression for mGlu8 in the rat basal ganglia was Striatum > GPe > STN  $\geq$  GPi > SNpr > SNpc.

Figure 2.4: Distribution of mGlu8 immunoreactivity in the rat basal ganglia



**Figure 2.4:** Photomicrographs showing the distribution of immunoreactivity for the mGlu8 receptor in the rodent basal ganglia. mGlu8 immunoreactivity is shown in (A) the striatum, (B) the external globus pallidus (GPe), (C) the entopeduncular nucleus (EP) or internal globus pallidus in primates and humans (GPe [arrow]), (D) the subthalamic nucleus (STN [arrow]) and (E) the substantia nigra pars compacta/ reticulata (SNpc [dashed arrow]/ SNpr [arrow]) and (F) no primary antibody control, showing immunoreactivity of the secondary anti-rabbit antibody in the substantia nigra. Arrows in the right hand panel indicate dorsal border of target nuclei. Scale bars equal 200µm.

## 2.5: Discussion

The preliminary immunohistochemical studies described in this chapter demonstrate that group III mGlu receptors mGlu4, 7 and 8 are widely expressed throughout the basal ganglia. The expression of mGlu4 and 7 within these basal ganglia nuclei has previously been demonstrated and our findings are largely in good agreement with these studies. It is important to note findings from these studies are deduced solely from qualitative analysis since this was not a hypothesis led study, rather an explorative investigation to determine the distribution patterns of group III mGlu receptor subtypes in the basal ganglia. Thus it was important not to miss contextual detail which can often be the case following quantitative analysis. Furthermore, statistical models were not required in studies of this number to explain the observed findings where distribution patterns in each of the target nuclei were absolute, rendering quantification irrelevant.

Analysis of the mGlu4 receptor revealed widespread distribution throughout the basal ganglia. Particularly intense staining was recorded in the striatum, GPe and the SNpr which is consistent with the findings of Corti *et al* (2002). In addition the clearly diffuse fibrous nature of the mGlu4 staining suggests a likely pre-synaptic, axon terminal location, which has previously been confirmed using electron-microscopy (Kosinski *et al.*, 1999; Bradley *et al.*, 1999; Corti *et al.*, 2002). Interestingly the immunohistochemical studies described here demonstrate particularly intense staining for mGlu4 in the SNpc, which together with previously identified moderate levels of mRNA expression in the STN may suggest mGlu4 receptors are localised on the subthalamonigral terminals (Messenger *et al.*, 2002). At the anterior-posterior position of the SN examined in these studies (-5.3mm AP from bregma), this specifically refers to the dorsal tier of the SNpc in which the main target of efferent neurones is the sensorimotor dorsal striatum (Francois *et al.*, 1999; Haber, 2003; Joel & Weiner, 2000). Therefore, taken together these findings indicate that mGlu4 receptors may be suitably positioned to be worthy targets for providing symptomatic relief in PD on the one hand based on immunoreactivity observed in the SNpr, but more relevant to this thesis, provide functional neuroprotection owing to its distribution in the SNpc.

In contrast to mGlu4, only moderate intensity staining for mGlu7 was seen in the striatum, however intense immunoreactivity was seen in the STN and SNpr, with lower, but still moderate intensity, in both the GPe and GPi. Of particular interest to these studies was the high mGlu7 immunoreactivity observed in the SNpc, notably within a medial region of the SNpc dorsal tier with little to no immunoreactivity in the lateral segment. The presence of mGlu7 in the SNpc would suggest mGlu7 receptors may be worthy of investigation for target potential in PD. However, it is unclear at this point how the regional distribution of these receptors exclusively to the medial dorsal tier of the SNpc may affect this possibility.

Of cause for concern was the punctate nature of the mGlu7 staining, demonstrating co-localisation with nuclei in numerous regions of the basal ganglia. Confirmation of this co-localisation with a nissl stain would have indicated immunoreactivity was largely in the dendrites of neurones where one could have assumed mGlu7 receptors exist predominantly post-synaptically. Indeed, very low levels of mGlu7 immunoreactivity have been found at post-synaptic dendritic sites in both the GP (Bradley *et al.*, 1999; Kosinski *et al.*, 1999) and striatum (Kosinski *et al.*, 1999). However, the extent of the nuclear binding seen here coupled with previous reports that demonstrate mGlu7 receptors exist largely pre-synaptically, we can assume the mGlu7 antibody used in these studies was non-specific. This drawback is discussed more at length in the latter part of this discussion. Nonetheless, the pattern of mGlu7 staining throughout the basal ganglia would suggest a degree of specific binding, demonstrating mGlu7 receptors are likely located in the STN and its target regions, the SNpr and SNpc. Thus mGlu7 receptors may also be worthy targets to provide functional neuroprotection in a rodent model of PD.

The findings from these preliminary studies demonstrate for the first time the presence of mGlu8 in each of the basal ganglia nuclei investigated. Although this data has not been quantitatively compared, the levels of mGlu8 expression seen here appeared to be considerably lower than that seen for both mGlu4 and 7. This pattern is in agreement with in-situ hybridisation studies performed previously in our laboratory, which reported particularly low levels of mGlu8 mRNA in the GPe, GPi and the SNpr (Messenger *et al.*, 2002). The relative levels of expression between the

*in situ* and present immunohistochemical studies correspond well in many instances. For example, the high levels of expression of mGlu8 mRNA previously reported within the cortex may account for the highest level of mGlu8 seen here within the striatum, assuming a pre-synaptic localisation of mGlu8 on corticostriatal terminals. Furthermore, the moderate mRNA levels within the striatum and STN could account for the low-moderate mGlu8 receptor expression within the SNpr, assuming they reside pre-synaptically on striatonigral or subthalamonigral terminals. It is also possible the mGlu8 immunoreactivity seen in the SNpr is due to mGlu8 receptors localised post-synaptically, and given the mGlu8 mRNA expression reported in the SNpr (Messenger *et al.*, 2002), this possibility cannot be ruled out. These suggestions of course remain speculative until complimentary EM studies are performed to determine if mGlu8 immunoreactivity seen here corresponds to pre- or postsynaptic locations in the SN. A key finding from these studies is the very low intensity staining of mGlu8 in the SNpc with only a small degree of staining observed in the medial region adjacent to the ventral tegmental area (VTA). Collectively the findings described above demonstrate mGlu8 is widely distributed throughout the basal ganglia although based on the low immunoreactivity observed in the nuclei of the subthalamonigral pathway is the least favoured group III mGlu receptor subtype in terms of target potential.

As mentioned, this was a preliminary investigation and was by no means a conclusive assessment of group III mGlu receptor expression in the basal ganglia. Consequently a number of limitations must be addressed here in performing small studies of this nature. For example, additional experiments would have been desirable to verify the pre-synaptic origin of each of the group III mGlu receptor subtypes, i.e. to investigate co-localisation with a pre-synaptic terminal marker such as synaptophysin or synaptic vesicle protein-2. An additional discrepancy was the punctate nature of the mGlu7 staining, demonstrating co-localisation with nuclei in numerous regions of the basal ganglia, suggesting a degree of non-specific binding with this antibody. This may explain why this commercial antibody was only predicted to bind to mGlu7 in rat tissue based on its high sequence homology with human cDNA and would account for the lack of studies reporting using this antibody in rats. Furthermore, antibodies produced against synthetic, rather than natural peptides representative of their specific

receptor structures, may also contribute to the lack of specificity seen here. Indeed these non-specific effects are not uncommon, and demonstrate immunohistochemical studies of this nature, are by themselves inconclusive. Future studies would seek to use alternative antibodies, supported with western blot analysis to confirm specificity of the antibody using a corresponding blocking peptide. A positive control would also have been of worth here to determine the relative immunoreactivity observed in these studies. Based on the abundant immunoreactivity to mGlu4 and mGlu7 reported in the human brain, the cerebellum may have been a desirable region to assess the relative immunoreactivity of the group III mGlu receptors in the basal ganglia (Human Protein Atlas).

Generally, very low dilution factors were required in these studies (typically 1:50) suggesting the affinity for the antigen with each of the antibodies tested was low. During washing with low affinity antibodies, dissociation from the epitope is more likely leading to a loss of staining and may account for the reduced immunoreactivity seen with mGlu8 in these studies. Additionally using paraffin embedded tissue, many antigens fail to survive the paraffin processing or the fixation process which may also have contributed to the reduced signal with the mGlu8 immunoreactive antibody. However these suggestions remain speculative until additional studies are carried out using alternative antibodies. Although frozen tissue offers much better antigen preservation, paraffin processing was favoured in these studies since the morphological detail and resolution of the frozen sections is usually considerably inferior to tissue that has been embedded during specimen processing. Nonetheless taking these considerations into account, the preliminary findings described here are in good agreement with previous reports describing mGlu4 and mGlu7 receptor distribution, and for the first time provides an insight into the distribution pattern of mGlu8 throughout the basal ganglia.

Despite the clear limitations of these studies, our findings from this preliminary investigation demonstrate group III mGlu receptors are widely distributed throughout the basal ganglia, of especial significance here each revealing expression in the SNpc. Although it has been shown these receptors exist on both the pre and post-synaptic terminal, electrophysiological studies have found no evidence for a post-synaptic

action of group III mGlu receptors (Valenti *et al.*, 2003). At the present time, therefore, focus remains fixed on pharmacologically targeting potential pre-synaptic receptors.

Whilst the findings described in this chapter along with considerable evidence in the literature demonstrate group III mGlu receptors are widely distributed through the BG, few studies have examined the state of mGlu receptor expression in the motor loop under parkinsonian conditions. Given the important role of group III mGlu receptors in modulating glutamatergic and GABAergic transmission within the BG, it is likely that any changes in the expression of these receptors under the pathological condition of PD may exacerbate the already aberrant transmission within the motor loop and contribute to symptom generation. On the contrary, it is also possible that an elevation in glutamate may lead to plasticity of group III mGlu receptors thereby acting as a potential compensatory mechanism. Clearly, changes in the expression of mGlu receptors under parkinsonian conditions would therefore have important consequences on the overall outcome of these elevations in transmitter release in the BG and on the therapeutic potential of targeting these receptors.

Remarkably, there remains only one study from our laboratory that has examined the state of mGlu receptor expression in the BG motor loop under parkinsonian conditions. This report revealed few alterations in group III mGlu receptor gene expression following a unilateral 6-OHDA lesion of the median forebrain bundle (MFB) with the exception of mGlu4, in which a significant reduction in mGlu4 mRNA in the striatum (~14% reduction with respect to intact hemisphere) was observed. However these studies did not confirm if the decline in mGlu4 mRNA expression translated into changes at the receptor protein level. Whilst this possibility would likely serve more to exacerbate, rather than provide a compensatory mechanism against the ensuing nigrostriatal degeneration, the decline in mGlu4 mRNA whilst significant remained relatively minor. Furthermore no alterations in mGlu7 or mGlu8 receptor expression were observed, thus the relative persistence of these mGlu receptor subtypes following nigrostriatal tract lesioning along with their robust expression in the subthalamonigral pathway, suggests activation of group III mGlu receptors may offer a promising approach to halt ongoing degeneration in PD.

The potential of pharmacologically targeting these receptors will be explored in subsequent chapters of this thesis.



### **Chapter 3:** Potential of targeting group III metabotropic glutamate receptors to provide functional neuroprotection in the 6-OHDA rat model of PD

### 3.1 Introduction

In Chapter 2 of this thesis we identified immunoreactivity for each of the group III mGlu receptors (with the exception of mGlu6) in the five major nuclei of the basal ganglia, demonstrating particularly intense staining for mGlu4 and mGlu7 in the SN. Furthermore studies in our laboratory have suggested an autoreceptor role for group III mGlu receptors in the rat SNpr whereby activation of these receptors has been shown to reverse reserpine-induced akinesia using broad spectrum agonists (Austin *et al.*, 2010). We have also shown that group III mGlu receptor activation using one of these broad spectrum agents, L-SOP, reduces glutamate release in the SN *in vivo*, suggesting these receptors may hold potential in combating certain aspects of the neurodegenerative processes in PD. Therefore studies in this chapter focused on the potential of targeting group III mGlu receptors to provide functional neuroprotection using the pathologically relevant unilateral 6-OHDA lesion rat model of PD.

As detailed in Chapter I of this thesis, PD is characterised by a progressive degeneration of nigrostriatal dopaminergic neurons. It is thought this degeneration is caused by a complex interplay of factors, with oxidative stress, mitochondrial dysfunction and failure of the ubiquitin-proteasome system all strongly implicated in the neurodegenerative process. There is also considerable evidence to point to a role for glutamate-mediated excitotoxicity in the ongoing degeneration of nigral dopaminergic neurones in PD. A key factor supporting this notion is that loss of striatal dopamine in the parkinsonian basal ganglia drives downstream changes in the indirect pathway, ultimately leading to over-activity of the glutamatergic STN. The complex changes in the basal ganglia circuitry which lead to hyperactivity in the STN was the subject of detailed discussion in Chapter I (section 1.3.3) and will not be discussed again here. Of particular importance to the studies in this chapter however, are the increased levels of glutamate within the STN target regions caused by STN over-activity. These include the basal ganglia output regions (SNpr/ GPi), which drive the motor symptoms of PD, GPe, PPN and crucially the SNpc (Smith & Bolam, 1990). The SNpc expresses ionotropic glutamate receptors of both AMPA and NMDA subtypes, whereby most of the excitatory transmission is thought to be mediated by the NMDA subtype (Christoffersen & Meltzer, 1995). Increased

glutamate in the SNpc may contribute to the pathology of PD, resulting from chronic depolarisation mediated by ionotropic glutamate receptors, and consequent excess  $\text{Ca}^{2+}$  influx through NMDA receptor activation once the  $\text{Mg}^{2+}$  block has been removed (Doble, 1999). High intracellular  $\text{Ca}^{2+}$  can lead to numerous cytotoxic effects, ultimately leading to cell death. For a more detailed account of excitotoxicity please refer back to Chapter I section 1.1.2.3.2. Additionally, mitochondrial dysfunction in the SNpc found in PD patients may also lead to ‘indirect’ excitotoxicity. In this instance, maintenance of membrane polarity by ATP-driven  $\text{Na}^+/\text{K}^+$  ATPase activity is lost as a result of compromised ATP production. The resulting membrane depolarisation and expulsion of voltage-gated  $\text{Mg}^{2+}$  block renders dopaminergic neurones vulnerable to physiological levels of glutamate (Greenamyre, 2001). It is important to stress here, glutamate-mediated excitotoxicity is not thought to be the primary cause of cell death in PD since the STN only becomes hyperactive following nigrostriatal degeneration and resulting alterations within the indirect pathway. However sustained excessive glutamate release may trigger the neurodegenerative progression within vulnerable dopaminergic neurones of the SNpc (Rodriguez *et al.*, 1998).

Treatment of PD to date has focused on dopamine replacement strategies using L-DOPA and/or dopamine agonists, which effectively ameliorate the motor symptoms in the early stages of PD. However prolonged use results in disabling motor side effects, such as dyskinesias (discussed at length in Chapter 1 section 1.4.1.1). Furthermore these dopamine replacement strategies fail to address the ongoing progression of nigral degeneration. In an attempt to address this limitation, it has been suggested that surgical or pharmacological therapies which reduce STN over-activity, or modulate glutamate receptors in the SNpc, may be neuroprotective and serve to halt the progressive neurodegeneration in PD. Indeed, surgical intervention by ablation or deep brain high-frequency stimulation (DBS) of the STN have been shown to be effective in reducing the motor symptoms of PD, although these procedures have not been demonstrated to be neuroprotective in humans. A study in MPTP-treated primates however, has demonstrated that STN-DBS or kainic acid lesioning of the STN was neuroprotective, with 20-24% more dopaminergic neurones remaining within the SNpc (Wallace *et al.*, 2007). The authors also suggested that

neuroprotection was mediated by a reduction in glutamate-mediated excitotoxicity. Of course, surgical procedures have many drawbacks as previously mentioned in Chapter 1, such as limited availability and post-operative complications since the majority of patients are elderly. Therefore there is an overwhelming need to devise a pharmacological approach to decrease the over-activity of the STN and resulting increase in glutamate release.

In terms of pharmacological manipulation of glutamatergic transmission in the SNpc, a number of pre-clinical rodent studies have shown blockade of NMDA receptors acts to reduce the firing rates of STN neurones and protect nigral neurones from degeneration (Allers *et al.*, 2005; Blandini *et al.*, 2001; Sonsalla *et al.*, 1998). Unfortunately the clinical usefulness of these compounds has been compromised by severe side effects including ataxia, sedation, psychotic effects and cognitive impairment (Lee *et al.*, 1999). Thus in recent years attention has focused more on the potential of targeting mGlu receptors, which provide an alternative approach to negatively modulate excessive glutamate release within the SNpc, as well as offering improved side effect profiles. These factors have led to mGlu receptors being considered as potential targets to provide neuroprotection in PD (Nicoletti *et al.*, 1996). Indeed blockade of the excitatory group I mGlu receptors and activation of inhibitory group II mGlu receptors, have shown significant neuroprotection in 6-OHDA lesioned rodents (Murray *et al.*, 2002; Vernon *et al.*, 2007a).

Group III mGlu receptors, the focus of studies in this thesis, are also worthy candidates to consider here given that they are expressed in the SNpc and appear to function as autoreceptors. As shown in Chapter 2, immunohistochemistry revealed the presence of each of the group III mGlu receptors in the SN (albeit to a lesser extent with mGlu8) revealing particularly high intensity staining of mGlu4 receptors in the SNpc. In addition our laboratory has shown that activation of presynaptic group III mGlu receptors using the broad spectrum agonists L-AP4 and L-SOP, inhibited depolarisation-evoked [<sup>3</sup>H]-D-aspartate release (non-metabolised analogue of glutamate) by 44 and 33% respectively in nigral slices *in vitro* (Austin *et al.*, 2010). Microdialysis revealed L-SOP also reduced glutamate release in the SNpr *in vivo* by 48% with respect to vehicle treated animals (Austin *et al.*, 2010). Furthermore initial

*in vitro* studies have revealed neuroprotective effects following activation of group III mGlu receptors. For example, L-AP4 (1-3 mM), demonstrated protection against NMDA-induced toxicity in mixed cultures of mouse cerebellar granule neurones (Lafon-Cazal *et al.*, 1999a). In addition, this study demonstrated that L-AP4 could inhibit the 60-fold increase in glutamate (with respect to baseline) induced by NMDA by more than 60%, suggesting this neuroprotective effect was a result of combating glutamate-mediated excitotoxicity.

To date, few studies have been performed to investigate the neuroprotective potential of targeting group III mGlu receptors in animal models of PD. In 2005 Vernon *et al.*, were the first to demonstrate *in vivo* that group III mGlu receptor activation mediated neuroprotection. In this study sub-chronic intranigral infusion of L-AP4 for 7 days in 6-OHDA-lesioned rats resulted in complete preservation of striatal dopamine levels in the lesioned hemisphere, although the number of TH positive cell bodies was improved to a lesser, but still significant extent (Vernon *et al.*, 2005). However, no behavioural tests were performed in this study to indentify whether neuroprotection at the biological level correlated to a functional preservation in motor behaviour. Therefore it is clear further investigation into the involvement of group III mGlu receptors in mediating neuroprotection in animal models of PD is required.

## 3.2 Aims

The overall aim of the studies described in this chapter was to further elucidate the potential of targeting group III mGlu receptors as an alternative, non-dopaminergic, therapy to provide neuroprotection in the treatment of PD. Initial studies sought to confirm the previously reported neuroprotective effects of L-AP4 in our 6-OHDA lesion model and to further investigate if this protection translated to preservation in motor function. We next investigated whether the effects of L-AP4 were indeed group III mGlu receptor mediated by examining whether the response to L-AP4 was lost following pre-treatment with a selective group III mGlu receptor antagonist CPPG.

More specifically, the key objectives here were:

1. to provide immunohistochemical and neurochemical confirmation of the neuroprotective effects of the broad spectrum group III mGlu receptor agonist L-AP4 against a unilateral 6-OHDA lesion of the SNpc in rats
2. to ascertain whether neuroprotective effects of L-AP4 translate to functional restoration of motor function in both forced and habitual behavioural tasks
3. to establish the effective dose range of L-AP4 to provide neuroprotection as determined by behavioural and immunohistochemical assessment
4. to determine if functional neuroprotection achieved with L-AP4 is reversed on pre-treatment with a selective group III mGlu receptor antagonist CPPG

### **3.3 Methods**

#### **3.3.1 General methodological considerations**

##### **3.3.1.1 6-OHDA-lesion model of Parkinson's disease**

To investigate the neuroprotective potential of targeting group III mGlu receptors on the subthalamonigral pathway it was essential to utilise a pre-clinical model of PD that effectively mimicked the hyperactivity in the STN of PD patients. As mentioned in section 1.3.3.2, the MPTP model has shown a degree of hyperactivity in the STN, reporting a 27% increase in STN firing rates in the MPTP primate (Bergman *et al.*, 1994). However following a 6-OHDA lesion of the SNpc in rats, huge increases in STN activity have been reported, with STN neurons demonstrating discharge rates exceeding 233.3% of non-lesioned animals (Magill *et al.*, 2001). This would suggest the 6-OHDA lesion model would provide an ideal test bed in which to investigate the potential of group III mGlu receptor ligands to mediate neuroprotection by inhibiting the hyperactivity in the STN. Therefore in the studies described in this thesis, the SNpc was chosen as the site of stereotaxic injection of 6-OHDA to induce degeneration of the dopaminergic nigrostriatal tract, although the striatum and the MFB are other common sites of administration.

Nigral or MFB administration causes rapid cell loss which begins within 12-24 hours, resulting in maximal striatal dopamine-depletion typically after 2-3 days (Faull & Lavery, 1969). Conversely, striatal injections of 6-OHDA lead to a more protracted retrograde degeneration of the dopaminergic neurones within the SNpc, taking between one to three weeks to develop a stable lesion (Przedborski *et al.*, 1995). The site of injection also has an impact on which dopaminergic cells degenerate. Lesioning of the MFB causes total destruction of both A9 nigral neurones, which project to the dorsolateral striatum and A10 mesolimbic neurones of the VTA, which project to the ventromedial striatum (Thomas *et al.*, 1994; van Domburg & Ten Donkelaar, 1991). Nigral administration of 6-OHDA favours degeneration of A9 cells, with a lesser degeneration of A10 neurones (Carman *et al.*, 1991). Therefore injection of 6-OHDA into the SNpc was the most suitable site for use within our

studies to test the neuroprotective ability of sub-chronic (8 days) administration of group III mGlu receptor ligands on A9 nigral neurones. Furthermore, this approach allowed a single cannulation site for both lesioning and drug administration.

Due to the small size of the SNpc there is often some variability in lesion size, therefore studies described in this thesis aimed for a minimum of 7 animals per group. The lesion size for these studies was based on a dose of 6-OHDA which induces a suitable deficit to measure changes in motor behaviour and is discussed further in section 3.3.1.2 on introduction of behavioural tests. To maintain specific destruction of dopaminergic neurones in these studies, the uptake-1 inhibitor, desipramine was given pre-lesion to prevent uptake of 6-OHDA into noradrenergic neurones. To enhance bioavailability of 6-OHDA within the brain, the monoamine oxidase-B inhibitor, pargyline was also given pre-lesion to inhibit 6-OHDA degradation.

When modelling PD with stereotactic injection of 6-OHDA, it is possible to induce bilateral lesions which are not affected by any compensation from the intact side, and therefore mimic the pathology of idiopathic PD more closely (Deumens *et al.*, 2002). Whilst bilateral lesions would have been desired for these studies, they are associated with a high level of mortality, with animals displaying extreme akinesia with aphagia (difficulty swallowing) and adipsia (deficit in drinking), requiring them to be tube-fed (Ungerstedt, 1971c). Therefore unilateral lesions are more commonly performed. Whilst bilateral 6-OHDA models are pathologically more relevant, unilateral lesion models allow for drug-induced rotations as behavioural indicators of lesion size, as well as maintaining the intact hemisphere to be used as a control for neurochemical and histological analyses. The ability to construct a unilateral lesion was an additional major factor in the selection of the 6-OHDA model for this thesis since it provides the opportunity to investigate functional neuroprotection by gauging the asymmetry between the lesioned and non-lesioned hemispheres. In addition the 6-OHDA lesion is quick, relatively inexpensive and reproducible in nature, and for these reasons remains one of the most regarded early pre-clinical models for the investigation of neuroprotective strategies for PD.



### 3.3.1.2 Behavioural tests

Asymmetry in the dopaminergic nigrostriatal tract following 6-OHDA lesioning leads to quantifiable differences in basal ganglia function between the two hemispheres. This can be exploited by assessing motor function asymmetries in habitual or forced motor tasks or by assessing drug-induced rotational behaviour. Habitual motor tasks which can test forelimb asymmetry include the cylinder test, where the animal is placed in a transparent cylinder, and during a fixed time period rearing behaviour is monitored for asymmetry of paw use. This test is a sensitive measure to determine the functional integrity of the nigrostriatal system since the animals are left to their own devices. Such a test is ideal for neuroprotection studies to provide an insightful measure of motor asymmetry following a 6-OHDA lesion and to assess functional preservation following treatment with potential neuroprotective agents. For this reason this test was employed in all neuroprotection studies described in this thesis. However in some cases if animals are heavily lesioned and demonstrate a high level of akinesia, the number of rearing behaviours against which to assess motor asymmetry can be compromised. Therefore a forced motor task such as the adjusted steps test was also employed in these studies, where an asymmetry score could be reliably attained.

The adjusted steps test is an examination of akinesia within each forepaw as the animal is moved slowly over a fixed distance along a bench, with only one forepaw bearing weight and adjusted steps counted. It has been shown that adjusted steps by the contralateral paw are profoundly reduced in both directions following 6-OHDA-lesioning, whereas the ipsilateral paw remains largely unaffected (Olsson *et al.*, 1995). Furthermore, the same study revealed this test was robust enough to emphasise functional recovery from a 6-OHDA lesion, with significant improvements in contralateral backhand adjusted steps following striatal and nigral ventral mesencephalon transplants (Olsson *et al.*, 1995). Therefore owing to its ability to identify motor asymmetries following 6-OHDA lesioning and detect improvements in adjusted steps following dopamine cell transplantation, as well as being relatively easy to perform, the adjusted steps test was selected to assess changes in motor asymmetry following treatment with group III mGlu receptor ligands in these studies.

Drug-induced rotational behaviour is commonly induced by two agents, the mixed D<sub>1</sub>/D<sub>2</sub> receptor agonist, apomorphine or the indirectly acting sympathomimetic, amphetamine. Dopaminergic denervation induces postsynaptic dopamine receptor supersensitivity in the lesioned striatal hemisphere. Apomorphine-mediated stimulation of these receptors is therefore enhanced in comparison to those on the intact side (Ungerstedt, 1971b) and the resultant unilateral overactivity induces turning away from the side of the lesion referred to as contraversive rotations. However, only when greater than 90% of the nigrostriatal cells have degenerated will a 1mg/kg dose of apomorphine induce rotational behaviour of greater than 4 rotations min<sup>-1</sup> (Hefti *et al.*, 1980). Conversely, an injection of amphetamine results in the release of vesicular dopamine predominantly from the intact hemisphere, where dopaminergic terminals remain, therefore inducing rotational behaviour towards the lesioned side (ipsiversive rotations, Ungerstedt, 1971a). A 5mg/kg dose of amphetamine induces rotational behaviour once 50% or more of the nigrostriatal cells are lost (Hefti *et al.*, 1980). Furthermore there is good correlation between the number of rotations and A9 nigral neurones lost for lesions between 50-100% (Kirik *et al.*, 1998; Moore *et al.*, 2001). In these studies, it was decided to use an almost full lesion size of 80-90%, in keeping with the neuroprotection studies of O'Neill *et al.*, (2004), who also examined drug-induced rotational behaviour. This provides a lesion of sufficient size to monitor graded improvements in motor function to assess neuroprotective effects of group III mGlu receptor agonist/modulators. Thus, amphetamine-induced rotations were deemed preferential to apomorphine rotations, in animals with an 80-90% lesion size, as amphetamine offers a graded rotational response proportional to lesion size in this range.

### **3.3.2 Specific methodological details for L-AP4 neuroprotection studies**

#### **3.3.2.1 Animals**

Male Sprague Dawley rats (B & K or Harlan, U.K.) weighing 270 – 300 g were used in these studies. Food and water were provided *ad libitum*. Animals were housed in a temperature- and humidity-controlled environment with a 12-h light / dark cycle. All procedures conformed to the U.K. Animals (Scientific Procedures) Act, 1986 and every effort was made to minimise animal numbers and suffering.

#### **3.3.2.2 Experimental protocol for surgical cannulation**

Animals were bilaterally cannulated so that in an event of blockage of one cannula the animal could still be used if the second cannula was patent. Two 12mm, 23 gauge stainless steel guide cannulae (Coopers), spaced 4mm apart were attached using a guide cannula holder, to a stereotactic micromanipulator (Kopf). Animals were subjected to isoflurane (Abbott animal health) general anesthesia (3-5% in 95% O<sub>2</sub>/5% CO<sub>2</sub>) in an induction chamber until they showed loss of pedal-withdrawal and corneal reflexes. Each animal was then placed into a small animal stereotactic frame (900 series, Kopf) with the incisor bar at -3.3mm below the interaural line and anesthesia was maintained at 2.5% isoflurane. The head was shaved and skin cleaned with an alcohol swab. A scalpel was used to make a rostrocaudal incision and the skin was held back using four artery clamps. The scalpel was then used to remove the periosteum from the skull, clearly exposing bregma, with excess blood removed with sterilized cotton buds and gauze. Three holes were then drilled through the skull (2 anterior and 1 posterior), clear of bregma and expected sites of cannulae implantation, then microscrews were screwed into the holes. The stereotactic location was marked for two further holes, which were then drilled to allow the cannulae to pass through the skull. Using the micromanipulator the cannulae were then stereotactically implanted bilaterally 2mm above both SNpc using the following co-ordinates; anterioposterior (AP), -4.8mm, mediolateral (ML),  $\pm$  2.0mm; dorsoventral (DV), -6.3mm from bregma (Paxinos & Watson, 1998). Cannulae were then secured into position using dental cement, which anchored the cannulae to the microscrews. The

cannulae holder was then withdrawn and a plastic collar (5mm section of a 1ml pipette tip) was secured into place with further dental cement, which protected the protruding cannulae ends from damage. Additionally, 30-gauge 12mm long stylets (Coopers) were placed into the cannulae to prevent blockage. A rehydrating solution of 0.9% saline with 0.1% glucose (5ml/kg, s.c., Baxter Healthcare) was administered and any loose skin surrounding the dental cement was sutured together. Following completion of surgery, animals were allowed to recover in clean cages placed on thermostatically heated mats until fully conscious.

### **3.3.2.3 Supranigral drug administration**

A minimum of 5 days following cannulae implantation, the neuroprotection study commenced. Animals received their first dose of L-AP4 (0.1-30nmol dissolved in 4µl of 1 x PBS) or vehicle (4µl 1 x PBS) 1 hour prior to 6-OHDA infusion (detailed in 3.3.2.4) and then once daily for 7 days post lesion. Since a severe lesion model was desired for these studies in which 6-OHDA is injected directly into the SNpc, degeneration is typically rapid. Unpublished findings from our collaborators at Eli Lilly have shown that degeneration of dopaminergic neurones in the SNpc begins 2 hours after injection of 6-OHDA, with a further report demonstrating the majority of cell death in the SNpc occurs within the first 3 days of injection (Zuch *et al.*, 2000). Thus, neuroprotective drug interventions are often, as in this case, introduced just prior to toxin injection to compensate for the acute nature of the 6-OHDA model. Furthermore, whilst the majority of degeneration likely occurs within the initial 3 days post lesion, Vernon *et al.*, (2007), have shown L-AP4 provides greater preservation of TH-positive cells in the SNpc following a 7 day treatment compared to a 3 day post-lesion treatment schedule. Consequently L-AP4 and all additional group III mGlu receptor agents were administered for 7 days post 6-OHDA lesion. Table 3.1 summarises the selectivity of L-AP4 and all other pharmacological agents administered supranigally in this thesis.

The doses of L-AP4 selected for these studies were based on a previous account investigating the neuroprotective profile of L-AP4 *in vivo* using a partial 6-OHDA lesion of the SNpc (Vernon *et al.*, 2007). In this study 10nmol/4µl L-AP4 was shown

to significantly protect TH-positive cell loss in the SNpc whilst this effect was lost at a higher (50nmol/4µl) dose of L-AP4. The neuroprotection studies described in this chapter, therefore, sought to extend these initial findings by implementing additional doses of L-AP4 to gauge a fuller dose response profile and seek to identify if protection provides preservation of motor function which to date remained unexplored.

Prior to infusion, the injection needle (13mm, 30-gauge stainless steel needle, Coopers), connecting tube (Portex) and 5µl microsyringe (Hamilton 7000 series) were filled with vehicle solution to ensure a closed system containing no air bubbles. Immediately prior to infusion, the plunger was pushed down, expelling vehicle solution, before the microsyringe was refilled with drug. After removing the stylet the injection needle was placed into the cannula so that the tip extended 1mm below the guide cannulae, reaching 1mm above the SNpc. Unilateral drug infusions were made into the right cannula (or left if the cannula was blocked) using a motorized micropump (KD Scientific), at a rate of  $2\mu\text{l min}^{-1}$  so that each injection took 2 min. Following infusion, the needle was left inside the cannula housing for 2 min to allow diffusion of the drug from the site of injection and avoid it being drawn back up the cannula. The stylet was then replaced into the cannula. Animals then received daily infusions of L-AP4 for the next 7 days, each being made into fully conscious animals as described here for the pre-lesion infusion.

In a separate study the neuroprotective effects of L-AP4 were investigated following pre-treatment with a broad spectrum group III mGlu receptor antagonist CPPG to confirm a receptor mediated process. This was achieved pharmacologically since there currently remain no group III mGlu receptor subtype knock out rats available. Thus to investigate the effects of CPPG on neuroprotection mediated by L-AP4, CPPG (75 nmol; dissolved in 4µl 0.1M NaOH) or its corresponding vehicle (4µl 0.1M NaOH) were infused at a rate of  $2\mu\text{l min}^{-1}$  unilaterally above the SNpc 30 min prior to each injection of L-AP4 using the same method described above.

	mGlu4	mGlu6	mGlu7	mGlu8	Reference
<b>Group III mGlu agonist</b>					
Glutamate	3-38	5-36	>1000	3-11	Cartmell & Schoepp., 2000
L-AP4	0.2-1	0.2-0.9	>100	0.06-0.09	Cartmell & Schoepp., 2000
DCPG	8.8	3.6	>100	<b>0.031</b>	Thomas <i>et al.</i> , 2001
<b>Group III mGlu allosteric modulator</b>					
VU0155041*	<b>0.7-0.8</b>	n.e	n.e	n.e	Niswender <i>et al.</i> , 2008
<b>Group III mGlu allosteric agonist</b>					
AMN082	>10	>10	<b>0.06-0.3</b>	>10	Mitsukawa <i>et al.</i> , 2005
<b>Group III mGlu antagonist</b>					
CPPG	2.2 against L-AP4-mediated inhibition of cAMP (no specificity to mGlu receptor subtype described)				Toms <i>et al.</i> , 1996

**Table 3.1.** Group III metabotropic glutamate receptor pharmacology: Potencies of agonists (EC<sub>50</sub> values) and antagonists (IC<sub>50</sub> values) at cloned human mGlu receptors (expressed in  $\mu$ M), highlighted in bold for the main group III target for each agent. \* VU0155041 also displays partial agonist activity with an EC<sub>50</sub> 2.5 $\mu$ M at mGlu4 receptors. n.e. = no effect observed with racemate of VU0155041 (VU0003423).

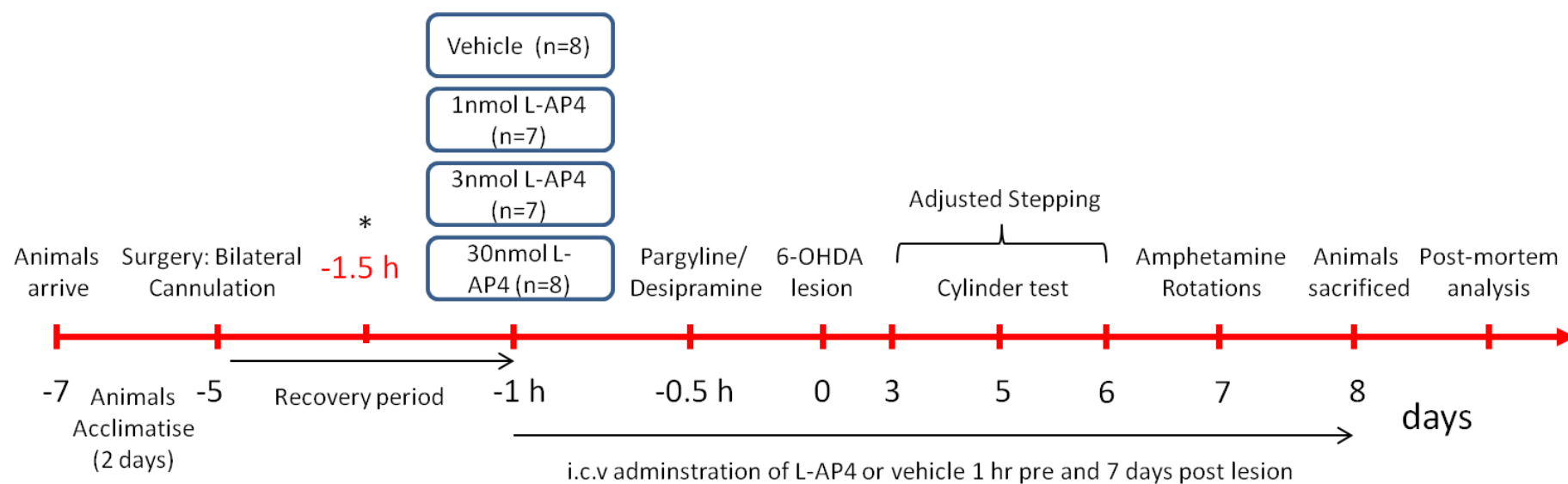
### 3.3.2.4 Intranigral 6-OHDA lesioning

Animals received an intranigral infusion of 6-OHDA (12 $\mu$ g) 1 hour after their supranigral infusion of L-AP4. 30 min prior to 6-OHDA infusion, animals were pre-treated with desipramine (25 mg/kg i.p.) and pargyline (5 mg/kg i.p.). A schematic diagram of the experimental design and timeline for L-AP4 and all further neuroprotection studies is shown in figure 3.1. To facilitate safe infusion of 6-OHDA, animals were then anaesthetized using isoflurane (Abbot animal health) general anaesthesia (3-5% in 95% O<sub>2</sub> / 5% CO<sub>2</sub>) in an induction chamber until they showed loss of pedal-withdrawal and corneal reflexes. Light anaesthesia was maintained at 1-

3% isoflurane during the 6-OHDA infusion. On the basis of requiring a suitably large enough lesion to measure changes in motor behaviour, and offering sufficient scope to assess neuroprotection, an 80-90% lesion was selected for these neuroprotection studies.

Initial studies in our laboratory investigating the dose-dependency of 6-OHDA (4-12 $\mu$ g), revealed a 12 $\mu$ g nigral infusion of 6-OHDA was deemed necessary to produce a lesion size of 80-90% (Austin, unpublished). As with supranigral delivery, prior to infusion, the injection needle (14mm, 30-gauge stainless steel needle, Coopers) connecting tubing (Portex) and 5 $\mu$ l micro syringe (Hamilton 7000 series, VWR) were filled with vehicle. Immediately prior to infusion, the plunger was pushed down, expelling vehicle solution, before the microsyringe was refilled with 6-OHDA. After removing the stylet, the injection needle was placed into the cannula so that the tip extended 2mm below the guide cannula, this time directly into the SNpc. Unilateral lesions of the SNpc (into the same hemisphere as L-AP4 given 1 hour earlier) were made by infusion of 6-OHDA (12 $\mu$ g dissolved in 2.5 $\mu$ l 0.02% ascorbic acid, in 0.9% saline, kept on ice and shielded from light) using a motorized micropump (KD Scientific). Infusions were made at a rate of 1.25 $\mu$ l min<sup>-1</sup> and therefore took a total of 2 min. Following infusion, the needle was left inside the cannula housing for 2 min, before withdrawal and then the stylet was replaced in the cannula. Upon completion of 6-OHDA infusion, animals were allowed to recover in clean cages placed on thermostatically heated mats until fully conscious.

Figure 3.1: Experimental design and timeline for L-AP4 and all further neuroprotection studies.



**Figure 3.1.** Schematic illustration of experimental design and timeline of events employed in L-AP4 neuroprotection studies. \* When required, CPPG or corresponding vehicle was administered 30 min prior to L-AP4 treatment.



### **3.3.2.5 Behavioural assessment**

The extent of motor impairment in animals following a 6-OHDA lesion was assessed at intervals using three behavioural tests: animal behaviour was assessed using the adjusted steps test on day 3 and 6 post-lesion, cylinder test on day 5 post-lesioning, (both tests also required a baseline assessment one day prior to lesioning) and amphetamine induced rotations on day 7 post-lesion.

#### *3.3.2.5.1 Cylinder Test*

Animals were placed individually in a transparent, perspex cylinder (21cm d x 34cm h) and the number of upward reaches video-recorded in 5-min intervals. Use of the ipsilateral and contralateral paw was then assessed as a percentage of total reaches made, both pre- and post-lesion, by observers blinded to treatment.

#### *3.3.2.5.2 Adjusted Stepping Test*

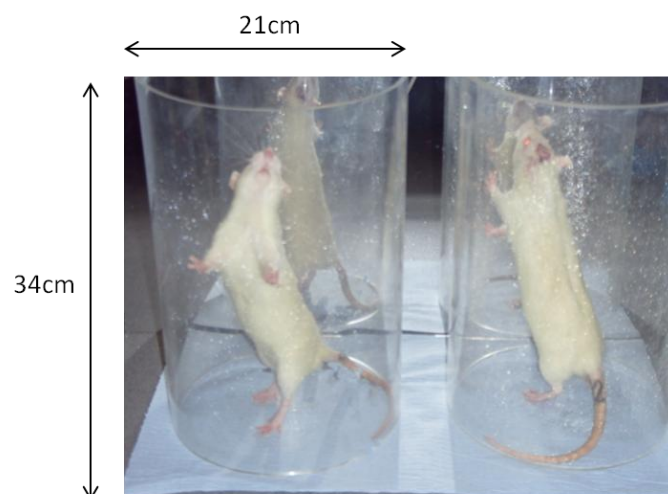
The rat was held in one hand, with the hind limbs gently restrained and the torso slightly raised and with the other hand, the forelimb that was not to be tested was also restrained. The rat was then moved slowly sideways in backhand and forehand directions across a 90cm distance with the free forelimb bearing the weight of the animal. The number of steps made by the animal were recorded in forehand and backhand directions, for both ipsilateral and contralateral paws. On each day of testing the adjusted steps test was repeated three times.

#### *3.3.2.5.3 Amphetamine-induced rotations*

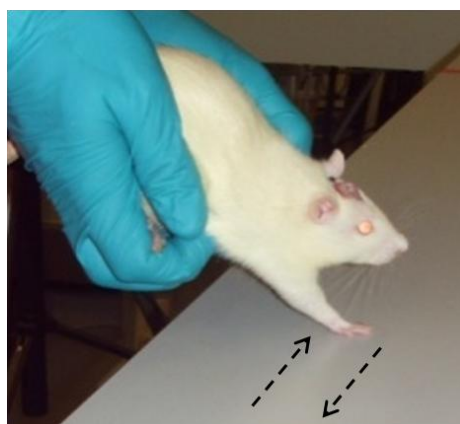
Amphetamine-induced rotational behaviour was assessed from video recorded observations of rats in 40cm diameter, flat-bottomed hemispherical bowls (Amee). After an initial 10 min baseline assessment period, animals received d-amphetamine (5mg/kg i.p.). Full 360° ipsiversive rotations were recorded in 5 min intervals for up to 60 min post injection and video tapes assessed by observers blinded to treatment.

Figure 3.2: Photomicrographs of behavioural tests employed in L-AP4 and all further neuroprotection studies.

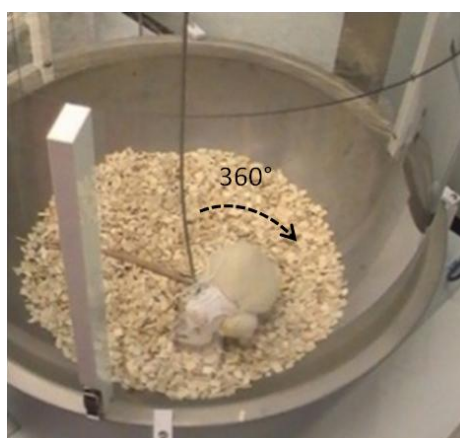
A)



B)



C)



**Figure 3.2.** Behavioural tests employed in neuroprotection studies. In chronological order, (A) cylinder test, (B) adjusted stepping and (C) amphetamine-induced rotations.

### 3.3.2.6 Data analysis for behavioural tests

Only behavioural data from animals showing correct cannula placement were included for subsequent data analysis. For the cylinder test, use of the ipsilateral and contralateral paw was assessed as a percentage of total reaches made, both pre- and post-lesion, by an observer blinded to treatment. Use of paw was then expressed as a percentage of total paw use for each animal. The mean percentage values were then obtained for each treatment group so that data are expressed as mean % paw use  $\pm$  standard error of the mean (s.e.m.). Data was then analysed using a 2-way ANOVA with Bonferroni's *post-hoc* test to determine whether there were significant differences between pre- and post-lesion scores between vehicle and L-AP4-treated animals.

The raw data of adjusted steps consisted of three replicates from each day of testing, which were then used to calculate an average value for both forehand and backhand directions for each paw. Average values of adjusted steps from 3 and 6 days post-lesion were then expressed as a percentage of the number of pre-lesion adjusted steps for each individual animal. The mean percentage values for each paw in each direction of movement at both 3 and 6 day time intervals were then obtained for each treatment group. Data are expressed as a mean %  $\pm$  standard error of the mean (s.e.m.). These data were then analysed using a 2-way ANOVA with Bonferroni's *post-hoc* test to determine whether there were significant differences between vehicle and L-AP4 treated animals at both time points tested.

Ipsiversive amphetamine-induced rotations were quantified in 5 min time intervals, with any contraversive rotations recorded as negative rotations. The number of ipsiversive rotations was plotted as both time course and total rotations in 60 min. Data are expressed as mean  $\pm$  standard error of the mean (s.e.m.). For time course data, a 2-way ANOVA with Bonferroni's *post-hoc* test was used to determine whether differences between vehicle and the effects of L-AP4 were significant at each time point. For total rotations in 60 min, the effects of L-AP4 treatment compared to vehicle were investigated using a 1-way ANOVA with Bonferroni's *post-hoc* test. In

all cases,  $P < 0.05$  was taken to be significant, with all statistical analysis performed using GraphPad Prism version 5.

### **3.3.2.7 Immunohistochemistry protocol**

On the final day of L-AP4 dosing (day 8), animals were terminally anaesthetized using pentobarbital ( $100 \text{ mg.kg}^{-1}$ ) then *trans*-cardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and cut into a rostral (striatal) and caudal (SN) segment and stored in PFA at  $4^{\circ}\text{C}$ .

Once required, the striatal and nigral segments of brain were dehydrated, defatted and then paraffin embedded.  $6\mu\text{m}$  coronal sections were taken through the striatum at +1.2, +0.2 and -0.26mm AP from bregma and through the SN at -4.8, -5.3, and -5.8mm AP from bregma (Paxinos & Watson, 1986) as described in section 2.3.2.2. Striatal and nigral sections were stained with diaminobenzidine (DAB) for tyrosine hydroxylase (TH) and dopa decarboxylase (DDC) as previously described in section 2.3.2.2 with the only alteration being the use of TH and DDC specific primary antibodies. TH is the first enzyme in the catecholamine biosynthesis pathway, catalyzing the conversion of L-tyrosine to L-DOPA and is therefore a useful marker of all catecholaminergic neurons. However in the striatum and SN, it is widely used a marker of dopaminergic cells since there are no noradrenergic neurons present. An additional marker of dopaminergic neurons, DDC, which catalyses the conversion of L-DOPA to dopamine was also investigated to confirm preserved levels of TH did not reflect increased expression of that marker.

Briefly, sections were de-paraffinised in xylene and 100% industrial methylated spirit (IMS) before a 10 min incubation in 3% hydrogen peroxide to block endogenous peroxide activity. Following standard antigen retrieval with boiling hot citric acid (1mM) then a 10-min incubation with blocking buffer (1% BSA in 0.5M TBS and 10% sodium azide, pH 7.6), sections were incubated for 2 hours at RT with rabbit- $\alpha$  - TH antibody (1:1250; Chemicon) or overnight at RT with rabbit polyclonal DDC antibody (1:1500; Chemicon). After washing in 0.1M TBS all sections were incubated with secondary antibody (goat anti-rabbit IgG-biotinylated 1:200, Sigma)

for 1 h at RT before washing again in 0.1M TBS. Following 30- min incubation with an ABC kit (Vector Labs) at RT, sections were washed in TBS and the signal developed in 0.05% DAB/0.03% H<sub>2</sub>O<sub>2</sub> solution dissolved in TBS (0.1M TBS, pH 7.6) for 10min at RT. Sections were finally dehydrated in 100% IMS, cleared in xylene, cover-slipped with DPX mountant and allowed to dry before being analysed using light microscopy.

### **3.3.2.8 Image and data analysis for immunohistochemical studies**

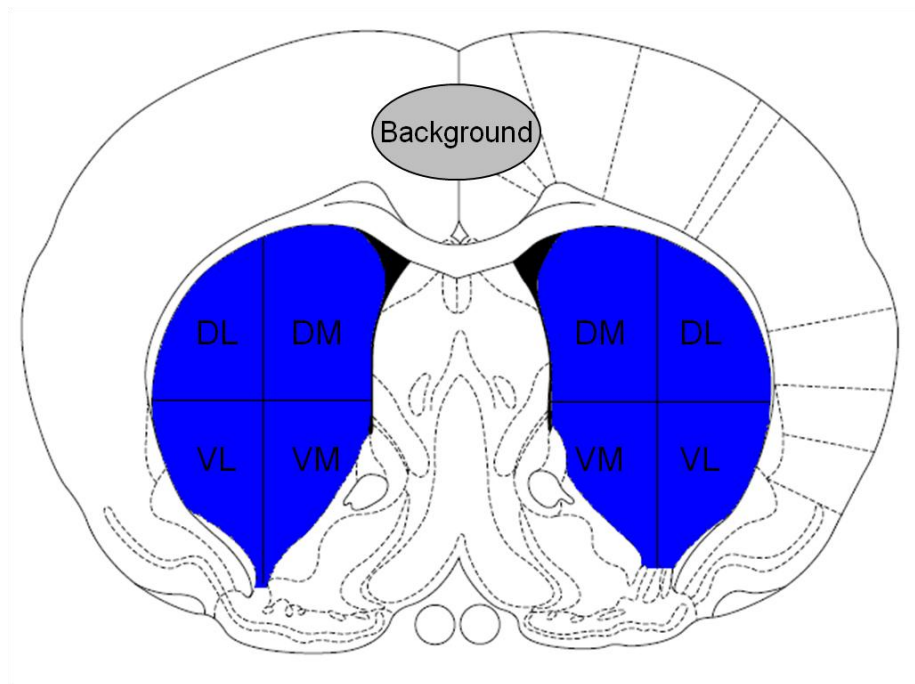
For TH and DDC immunohistochemistry in the striatum, digital images were captured on a mounted CCD Nikon camera, using Scion Image Software (Scion Corporation) at all three striatal regions. Initially TH optical density was recorded in four quadrants of the striatum and a background cortical region, the latter of which was subtracted from striatal readings (shown in figure 3.3). In order to control for variations in staining intensity between sections, the density of TH in each quadrant of the lesioned striatum was expressed as a percentage of that in the respective contralateral (intact) striatum. For DDC and further analysis of TH immunohistochemistry, the average of all four quadrants combined was also calculated to give an average value for the total striatal optical density of the intact hemisphere compared to the lesioned hemisphere. This process was repeated in triplicate sections from each animal to ultimately attain mean data for all rats per treatment group. The mean optical density of the 6-OHDA lesioned side was expressed as % of the contralateral, intact striatal hemisphere and was compared between treatment groups using a 1-way ANOVA with Bonferroni's *post-hoc* test.

For the SN, TH staining results were captured on a Zeiss apotome microscope and recorded using Axiovision LE software (Carl Zeiss Ltd., UK). TH-immunoreactive cells were counted in the contralateral and ipsilateral hemispheres of the SNpc in three adjacent sections for each animal across the three rostrocaudal levels and the mean of these values taken per treatment group. TH-positive cells were counted in regions of the SNpc which were clearly separated from the VTA by the medial terminal nucleus (MT) of the accessory optic tract. Within the SNpc, viable TH positive cells were counted at x50 magnification using image analysis software

(Image J). Since TH staining can be achieved in non-viable cells and sections were not stained for nuclei, only intact round cells displaying a clear nucleus and cytoplasm were included in these analyses. Thus, for each animal a total mean TH-positive cell count for the lesioned ipsilateral and contralateral hemispheres was obtained and expressed as a % of the contralateral, intact hemisphere. Statistical comparisons were made between L-AP4 and vehicle-treated groups using a 1-way ANOVA with a Bonferroni *post-hoc* test. All data are expressed as mean  $\pm$  standard error of the mean (s.e.m.), where *n* represents the number of animals in each experimental group. Statistical analyses were performed using GraphPad Prism (version 5.0) and in all cases  $P < 0.05$  was taken to indicate significance.

Stereological counting techniques provide an unbiased alternative method to assess neuroprotection in disease studies, whereby the reference volume may undergo substantial nonlinear changes as a function of age and/or disease state (due to *in vivo* size changes, as well as differential shrinkage during tissue processing), rendering this approach desirable to counteract these effects. However, a recent report comparing manual counting with stereological analysis revealed in a neuroprotection study using the same 6-OHDA lesion rat model, no difference in the outcome of these two measures, supporting the validity of our analysis (Iczewicz *et al.*, 2010). Consequently manual counting was performed in all neuroprotection studies detailed in this thesis.

Figure 3.3. Schematic illustration of striatal quadrants in which TH staining was quantified.

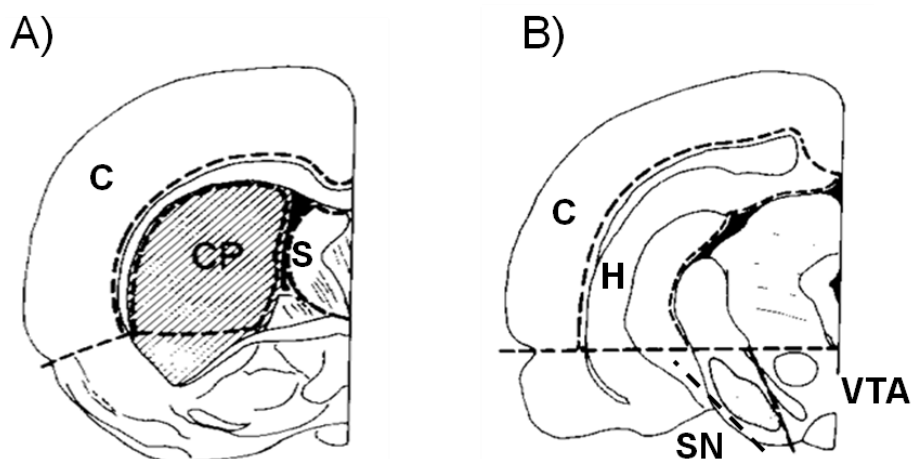


**Figure 3.3.** Schematic illustrating striatal quadrants in which TH optical density was measured, shown here at +0.2mm anteroposterior (AP) from bregma (Paxinos & Watson., 1998). A background reading was taken from the cortex (shown in grey) and subtracted from the striatal readings taken from each quadrant of both lesioned and non-lesioned hemispheres (shown in blue). DM: Dorsomedial; DL: Dorsolateral; VM: Ventromedial; VL: Ventrolateral.

### 3.3.2.9 Protocol for neurochemical assessment of dopamine

On the final day of L-AP4 dosing (day 8), animals were killed by CO<sub>2</sub> asphyxiation and brains were quickly removed for dissection of striatum and SN. Initially the brain was placed on its dorsal surface in the trough of a rat brain cutting block which was kept cold on crushed ice. Razor blades (kept on ice) were carefully inserted through the 1mm thick cutting channels of the block, slicing the brain at right angles to the sagittal axis. The brain was positioned so that an initial razor blade could be inserted tangential to the most posterior aspects of the olfactory tubercles as described by Heffner *et al.*, 1980. At this position the initial blade slices through the sagittal plane of the brain at the level of the body of the anterior commissure. The position of the first razor blade served as a reference point from which slices of the striatum and SN were subsequently taken. For dissection of the striatum, two additional razor blades were inserted anterior to the initial blade, along the rostral extent of the brain at intervals of 2mm. For the SN, two further razor blades were inserted posterior to the initial blade at intervals of 2mm. The razor blades were then removed from the block with the coronal brain slices adhering to their surfaces and placed on a glass petri dish containing dry ice. Using fine forceps the striatum was then dissected from these slices based on its distinct striated morphological appearance comprising tissue dorsal to the anterior commissure, ventral to the corpus callosum and medial to the external capsule. The stria terminalis was also removed from the ventrolateral borders of the septum (shown in figure 3.4A). Dissection of the SN was made from the furthest posterior brain slice from the initial cut in which the dark oval shape of the nigra was clearly visible on the ventrolateral portion of this section. A horizontal cut was then made across the brainstem at the level of the rhinal sulcus and dorsal edge of the SN (shown in figure 3.4B). After removing the telencephalic tissue lateral to the brainstem, tissue from the SN was obtained by extending ventromedially-directed cuts to the base of the brainstem to separate the VTA medial to the SN and ventral to the horizontal cut. Tissue was dissected bilaterally for both the striatum and SN and on completion was immediately placed in 1.5ml microtubes, weighed and placed in dry ice to be transferred to a -80°C freezer.





**Figure 3.4.** Diagrammatic representation of coronal sections from which the striatum/ caudate putamen (**A**) and substantia nigra (**B**) were dissected out. Dashed lines indicate boundaries of brain regions separated with forceps. C: Cortex; CP: Caudate putamen; S: Septum; H: Hippocampus; SN; Substantia nigra; VTA: Ventral tegmental area (figure adapted from Heffner *et al.*, 1980).

Striatal and SN samples were removed from frozen storage, homogenised in ice-cold buffer (0.1M PCA, 0.1mM EDTA, 2.5mg/L ascorbate) for approximately 4 sec and then centrifuged at 20,000g for 15 min at 4°C. The supernatant was then extracted and subsequently filtered in preparation to be assayed for dopamine by high performance liquid chromatography (HPLC) with electrochemical detection.

The HPLC system constituted a phase reservoir, on-line degasser (X-act degassing unit, Jour research, Sweden), pump (Rheos 4000, Flux Instruments AG, Switzerland) (flow rate 200µl/min), pulse dampener (Link, Antec Leyden, Holland) and an analytical column (150 x 2.1 mm; 3mm C18, Hypersil BDS). Samples were injected by means of an autosampler (Triathlon, Presearch, UK). Analytes were detected at 35°C by means of an electrochemical detector (Presearch, UK) with a thin layer electrochemical cell fitted with a glassy carbon working electrode maintained at +0.75V against a Ag / AgCl reference electrode. The mobile phase consisted of 100 mM sodium dihydrogen orthophosphate (NaH<sub>2</sub>PO<sub>4</sub>) and 100mM phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) mixed until a pH of 2.6 was obtained, added to 2mM 1-octane sulphonic acid (OSA), 1mM EDTA, and 13% methanol to reach a final pH of 2.8 required to achieve separation of analytes.. The flow rate was 200µl min<sup>-1</sup> and the limit of detection was approximately 0.1nM.

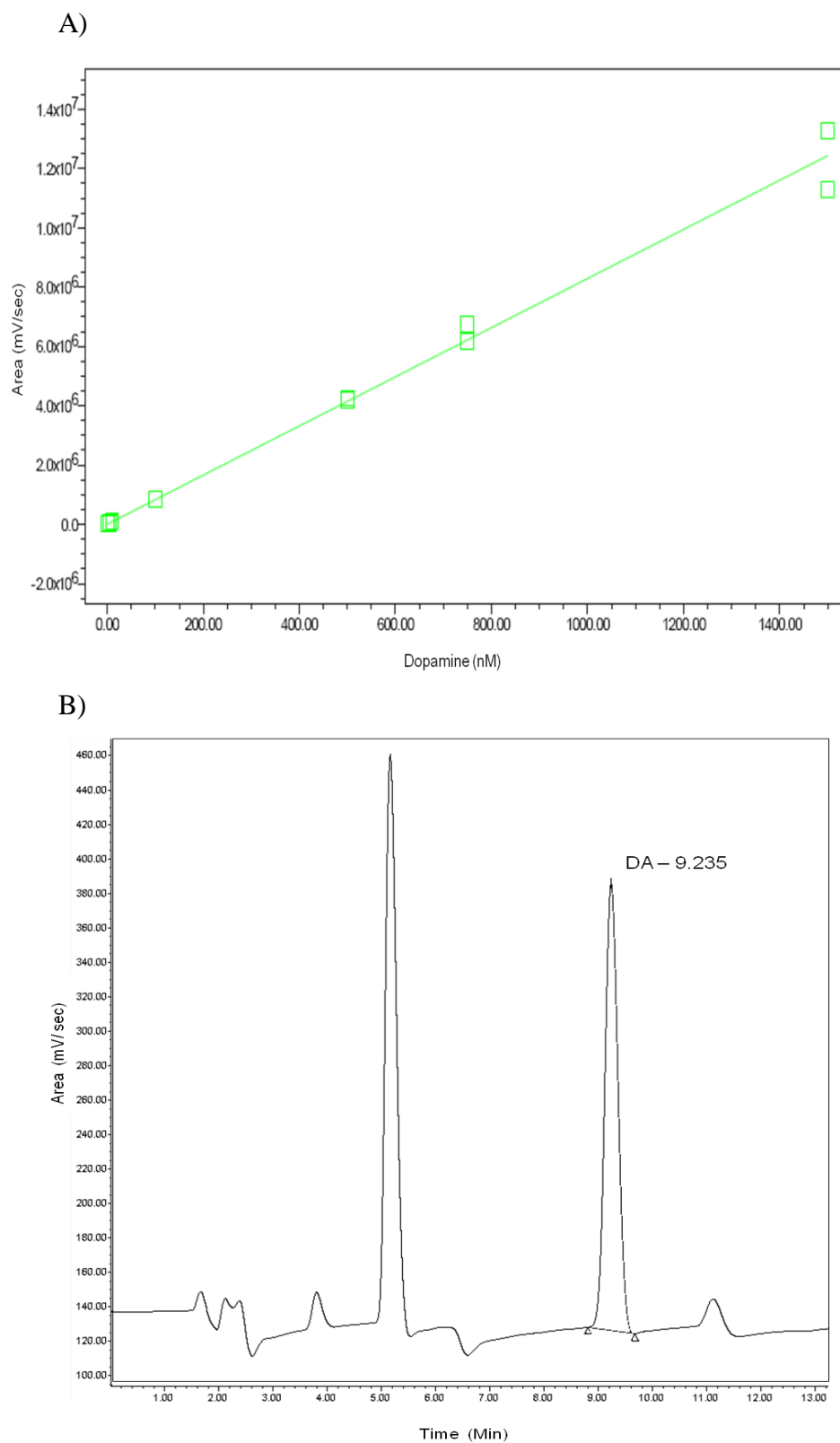
### **3.3.2.10 Data analysis for neurochemical studies**

Chromatogram analysis was carried out using Millenium<sup>32</sup> software (Version 3.05, Waters Corporation). Prior to the analysis of tissue dopamine, a standard curve was produced by running a series of standard solutions, containing known quantities of analyte dopamine, through the HPLC system at concentrations of 5, 10, 100, 500, 750 and 1500nM. From the resultant chromatograms a standard curve, such as the one shown in figure 3.5A was produced. Using Millenium<sup>32</sup> software, the peak areas were selected manually and the concentration (nM) of dopamine was then quantified by calibration of the selected peak area with the appropriate standard curve. Figure 3.5B illustrates a typical chromatogram profile in the reduction channel of dopamine in the striatum. A 1-way ANOVA with Bonferroni's *post-hoc* test was used to compare dopamine content of the 6-OHDA lesioned side, expressed as a % of the contralateral intact side between treatment groups.

### **3.3.2.11 Materials and suppliers**

Details of all reagents, consumables and supplier contacts are included in Appendix I.

Figure 3.5. Typical standard calibration plot and chromatogram for dopamine.



**Figure 3.5.** (A) Example of standard calibration curve for dopamine produced by analysis of 5, 10, 100, 500, 750, and 1500 nM dopamine standards run before and after each sample set to attain duplicate readings for each standard. (B) Example of a typical chromatogram illustrating striatal levels of dopamine (DA). Retention time (in min) is noted for dopamine peak.

### **3.3 Results**

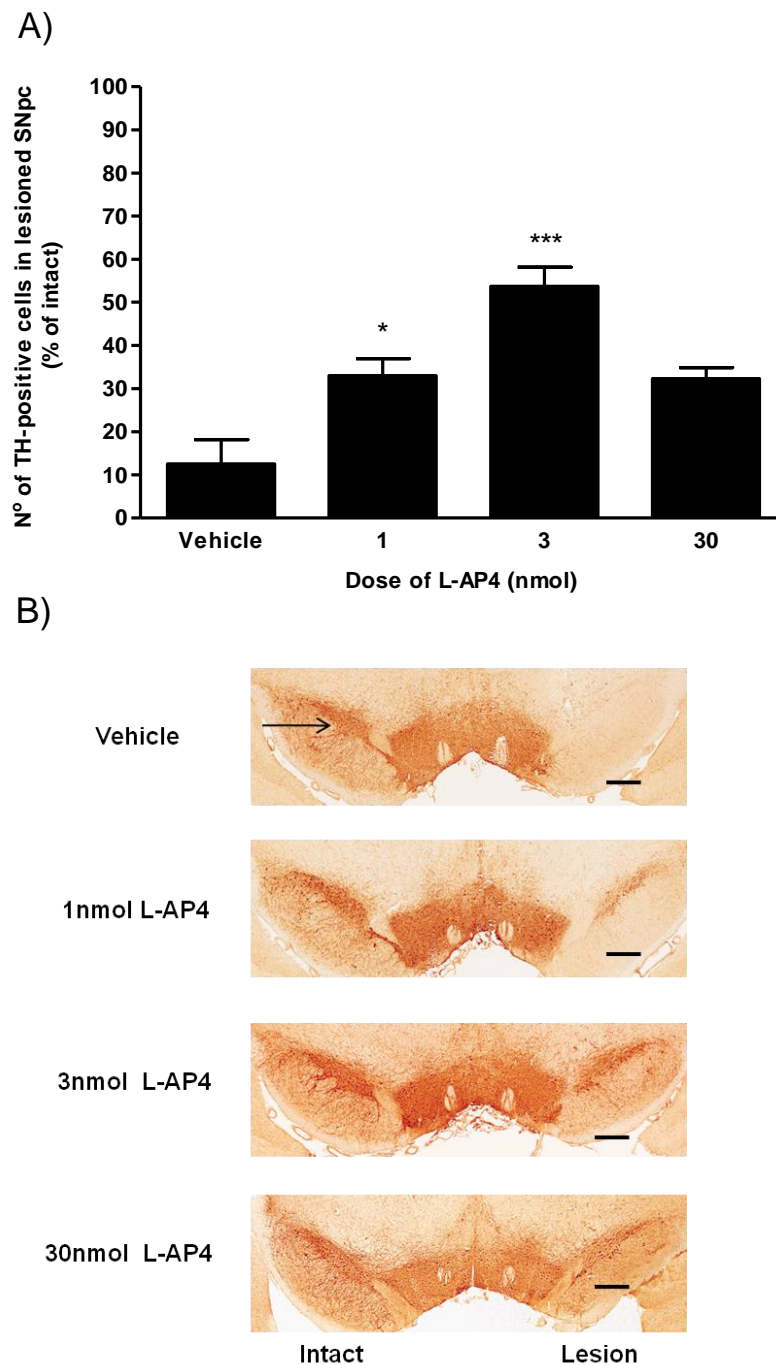
#### **3.3.1 Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist, L-AP4, in unilaterally 6-OHDA-lesioned rats**

##### **3.3.1.1 Effects of sub-chronic L-AP4 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta**

Within the SNpc, the mean sum of TH-positive cells in triplicate sections obtained across three rostrocaudal levels declined from  $307.4 \pm 19.1$  in the intact hemisphere, to  $36.9 \pm 16.1$  in the lesioned hemisphere of vehicle-treated 6-OHDA lesioned animals, a reduction of approximately 87%. Supranigral infusion of a low dose of L-AP4 (3nmol), 1 hour prior and for 7 days after 6-OHDA infusion demonstrated noticeable preservation of nigral TH-positive cells in the lesioned hemisphere. Following L-AP4 (3nmol) treatment the number of TH-positive cells remaining in the lesioned hemisphere amounted to  $53.7 \pm 4.4\%$  of the intact hemisphere, compared to an equivalent  $12.6 \pm 5.6\%$  remaining in vehicle-treated animals ( $P < 0.05$ ; 1-way ANOVA with Bonferroni's *post-hoc* test; figure 3.6A). Using a higher dose of L-AP4 (30nmol), preservation of nigral TH-positive cells was lost completing a bell-shaped dose response profile following sub-chronic supranigral infusion of L-AP4.

Representative nigral sections demonstrating immunoreactive TH-positive cells in the 6-OHDA lesioned animals treated with sub-chronic L-AP4 (1-30nmol) are shown in figure 3.6B. The photomicrographs demonstrate supranigral infusion of L-AP4 (3nmol) clearly provides noticeable preservation of TH-positive cells in the lesioned hemisphere. This effect appeared to be lost at the lower (1nmol) and higher (30nmol) dose of L-AP4 when compared to vehicle treated animals.

Figure 3.6: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on tyrosine hydroxylase (TH)-positive cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.



**Figure 3.6. (A)** The effects of sub-chronic supranigral infusion of L-AP4 (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 1x PBS) on the 6-OHDA induced loss of TH-positive cells in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=7 for 1 and 3nmol L-AP4, n=8 for 30nmol L-AP4). \* ( $P < 0.05$ ) and \*\*\* ( $P < 0.001$ ) indicate significant differences compared to vehicle treated animals (1-way ANOVA with Bonferroni's *post-hoc* test).  $F = 15.33$ ; degrees of freedom (df) = 3 (treatment), 26 (residual). **(B)** Representative photomicrographs show the levels of TH immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic L-AP4 treatment. Fixed coronal sections shown here are at -5.30mm AP from bregma, (Paxinos, 1998). Lesion side on the right; scale bar: 200 $\mu$ m

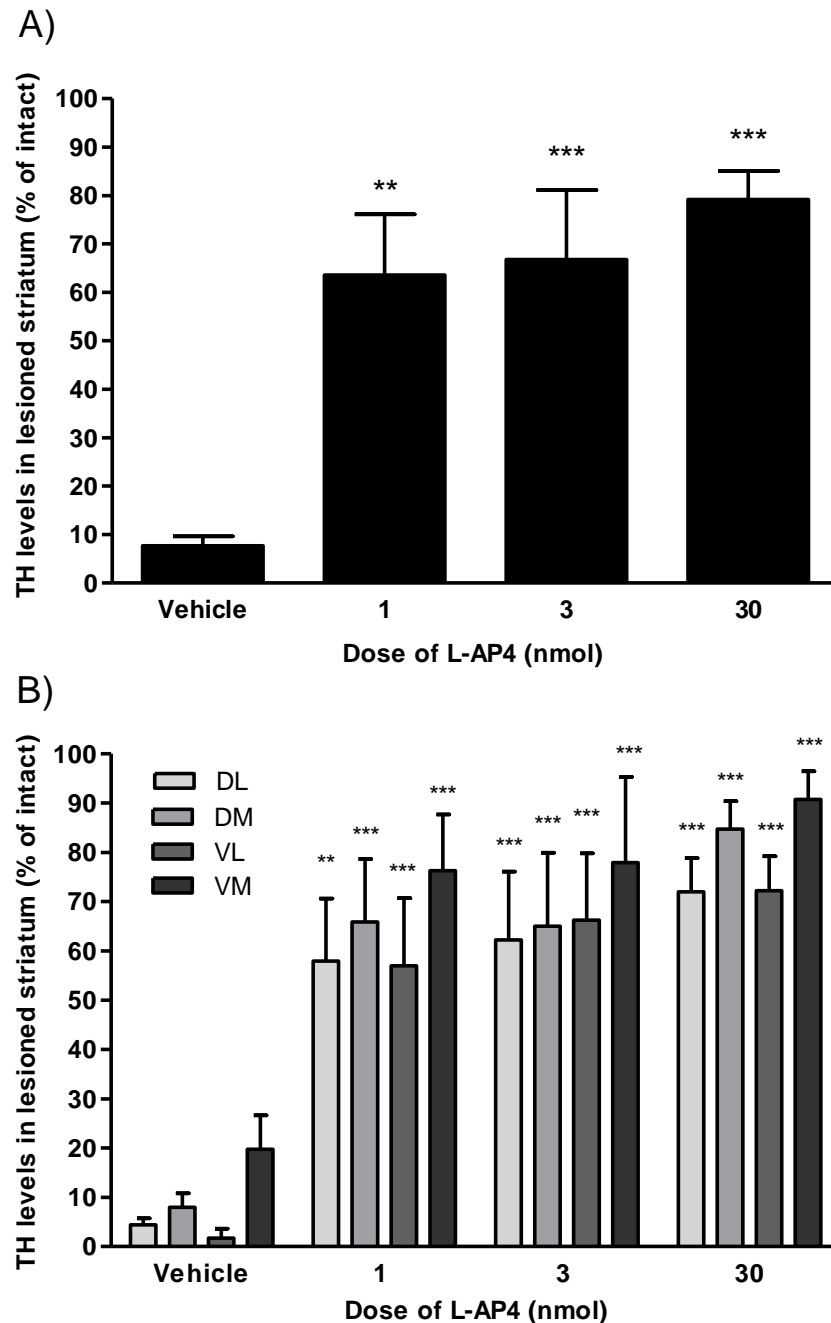
### **3.3.1.2 Effects of sub-chronic L-AP4 infusion on tyrosine hydroxylase levels in the striatum**

The optical density measurements of TH immunoreactivity in striatal sections from animals treated supranigrally with sub-chronic L-AP4 (1-30nmol) and those treated with vehicle is quantified in figure 3.7. When the combined mean striatal TH optical density was quantified, TH levels declined in the lesioned striatum of subsequent vehicle-treated animals to  $7.7 \pm 1.9\%$  of the intact side. Supranigral infusion of all doses tested of L-AP4 (1-30nmol, 1 hour prior and for 7 days after 6-OHDA infusion considerably preserved striatal immunoreactivity in the lesioned hemisphere. TH levels remained significantly higher ( $>63\%$  of intact side) in all L-AP4 treatment groups ( $n=7-8$ ;  $P<0.01$  versus vehicle treatment; 1-way ANOVA with Bonferroni's *post-hoc* test; figure 3.7A). Maximum protection was observed following 30nmol L-AP4 where the overall mean optical density in the lesioned striatum amounted to  $79.2 \pm 5.9\%$  of the intact hemisphere, suggesting considerable preservation of dopaminergic striatal nerve terminals in these animals.

When the individual quadrants of the striatum were quantified, each group showed a comparable level of TH immunoreactivity between quadrants ( $P > 0.05$ ) and again the levels were significantly preserved following L-AP4 treatment at each dose tested (figure 3.7B). Although assessment of TH in quadrants of vehicle treated animals revealed a tendency for greater loss of TH immunoreactivity in the dorsolateral and ventrolateral quadrants i.e. 96% and 98% loss in comparison to 92% and 80% loss in the dorsomedial and ventromedial quadrants respectively, this difference was not statistically significant ( $p > 0.05$ ; 1-way ANOVA with Bonferroni's *post-hoc* test).

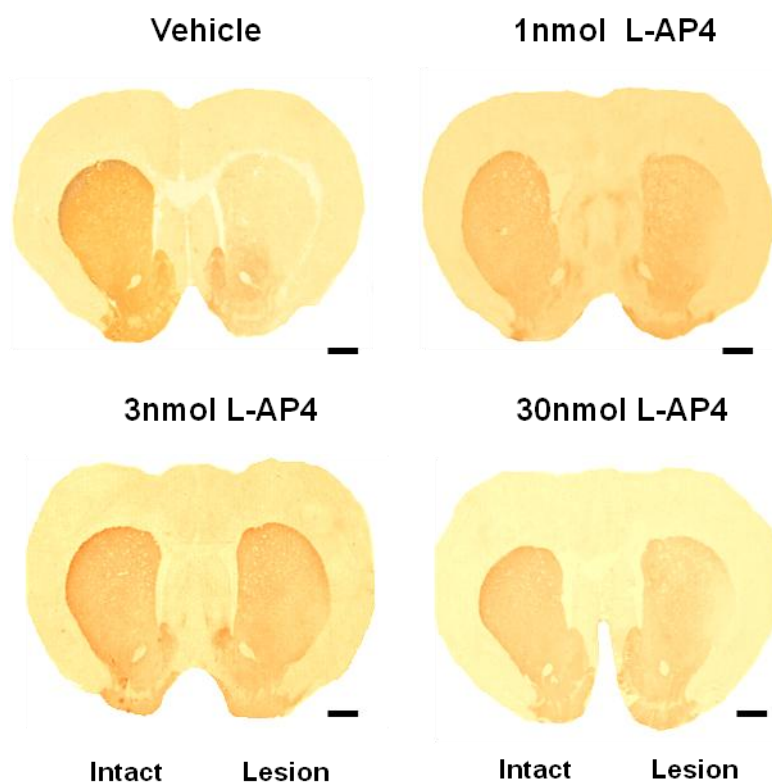
Representative striatal sections showing TH immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic L-AP4 (1-30nmol) are shown in figure 3.8. The photomicrographs demonstrate L-AP4 at each of the doses tested here provides considerable protection of striatal immunoreactivity when compared to vehicle treated animals.

Figure 3.7: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on tyrosine hydroxylase (TH) immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



**Figure 3.7.** The effects of sub-chronic supranigral infusion of L-AP4 (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of TH immunoreactivity in the whole striatum (**A**) and individual quadrants (**B**) on the lesioned side expressed as a percentage of the intact side. In (**A**), \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ) indicates significant difference compared to vehicle treated animals (1-way ANOVA with Bonferroni's *post-hoc* test).  $F = 15.33$ ;  $df = 3,26$ . In (**B**) \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ) indicate significant differences compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). For treatment factor,  $F = 43.15$ ,  $df = 3,104$ ; for quadrant factor  $F = 2.575$ ,  $df = 3,104$ . DL: Dorsolateral; DM: Dorsomedial; VL: Ventrolateral; VM: Ventromedial. For (**A**) and (**B**) values represent mean  $\pm$  s.e.m. ( $n=8$  for vehicle,  $n=7$  for 1 and 3nmol L-AP4,  $n=8$  for 30nmol L-AP4).

Figure 3.8: Tyrosine hydroxylase immunoreactivity in representative striatal sections from 6-OHDA-lesioned rats following sub-chronic treatment with the broad spectrum group III mGlu agonist, L-AP4.



**Figure 3.8.** Representative photomicrographs showing the levels of TH immunoreactivity in striatal sections from 6-OHDA-lesioned animals following sub-chronic L-AP4 treatment. Fixed coronal sections (shown here + 0.20mm AP from bregma, Paxinos, 1998) were reacted with antibodies raised against TH and stained with diaminobenzidine (DAB). Lesion side on the right; scale bar: 500 $\mu$ m.

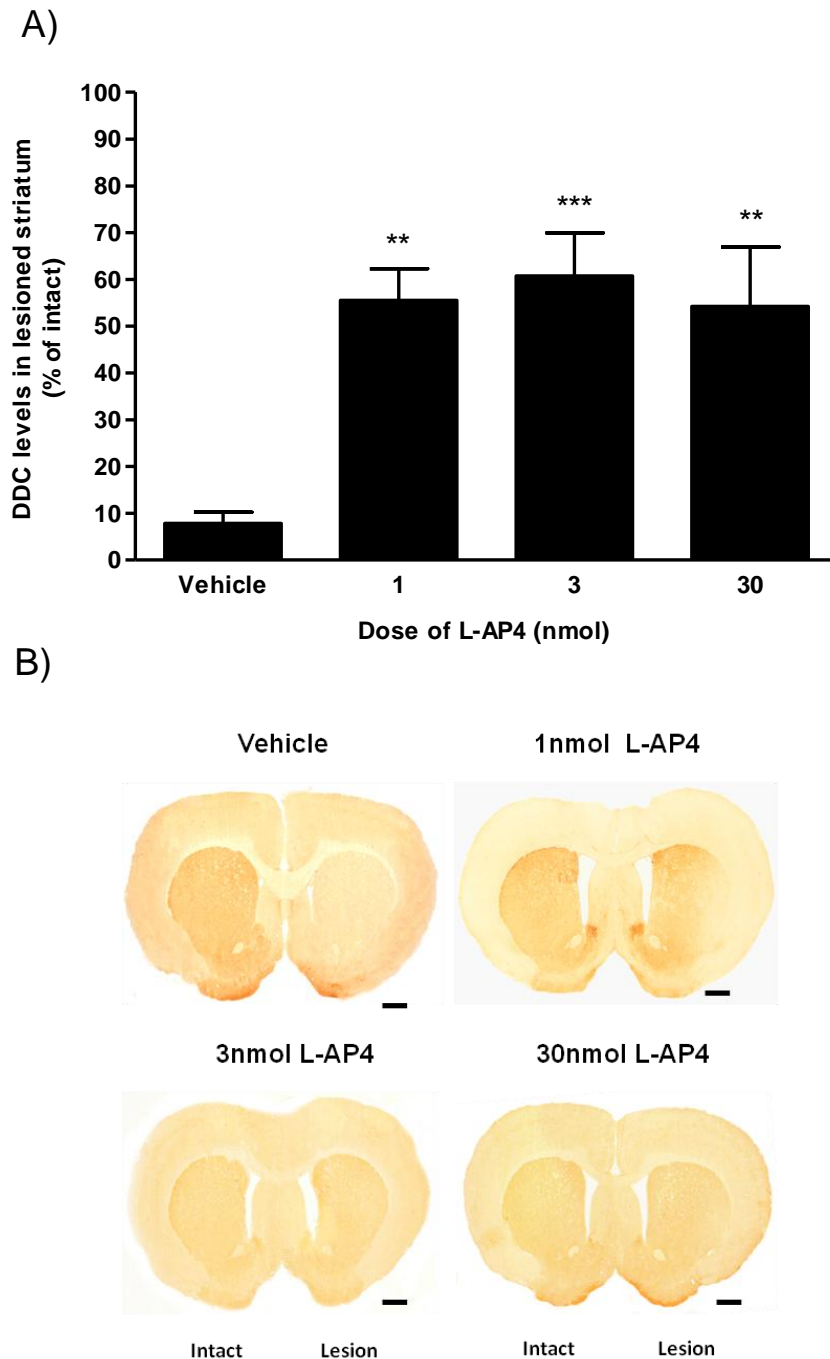


### **3.3.1.3 Effects of sub-chronic L-AP4 infusion on dopa decarboxylase in the striatum of 6-OHDA lesioned rats**

The optical density of DDC immunoreactivity in striatal sections from animals treated supranigrally with sub-chronic L-AP4 (1-30nmol) and those treated with vehicle are quantified in figure 3.9A. When the combined mean striatal DDC optical density was quantified, DDC levels declined in the lesioned striatum of subsequent vehicle-treated animals to  $7.9 \pm 2.4\%$  of the intact side. Supranigral infusion of all doses tested of L-AP4 (1-30nmol, 1 hour prior and for 7 days after 6-OHDA infusion considerably preserved striatal immunoreactivity in the lesioned hemisphere. DDC levels remained significantly higher ( $>54\%$  of intact side) in all L-AP4 treatment groups ( $n=7-8$ ;  $P<0.01$  versus vehicle treatment; 1-way ANOVA with Bonferroni's *post-hoc* test; figure 3.9A). Maximum protection was observed following 3nmol L-AP4 where the overall mean optical density in the lesioned striatum amounted to  $60.8 \pm 9.2\%$  of the intact hemisphere, demonstrating striatal DDC immunoreactivity was also significantly preserved with respect to vehicle-treated animals. This finding confirmed preservation of dopaminergic striatal nerve terminals in these animals and that preserved levels of TH was not a mere reflection of increased expression of the marker.

Representative striatal sections showing DDC immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic L-AP4 (1-30nmol) are shown in figure 3.9B. The photomicrographs demonstrate supranigral infusion of L-AP4 at each of the doses tested, 1 hour and for 7 days after 6-OHDA infusion provided noticeable protection of striatal immunoreactivity when compared to vehicle treated animals.

Figure 3.9: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on dopa decarboxylase immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



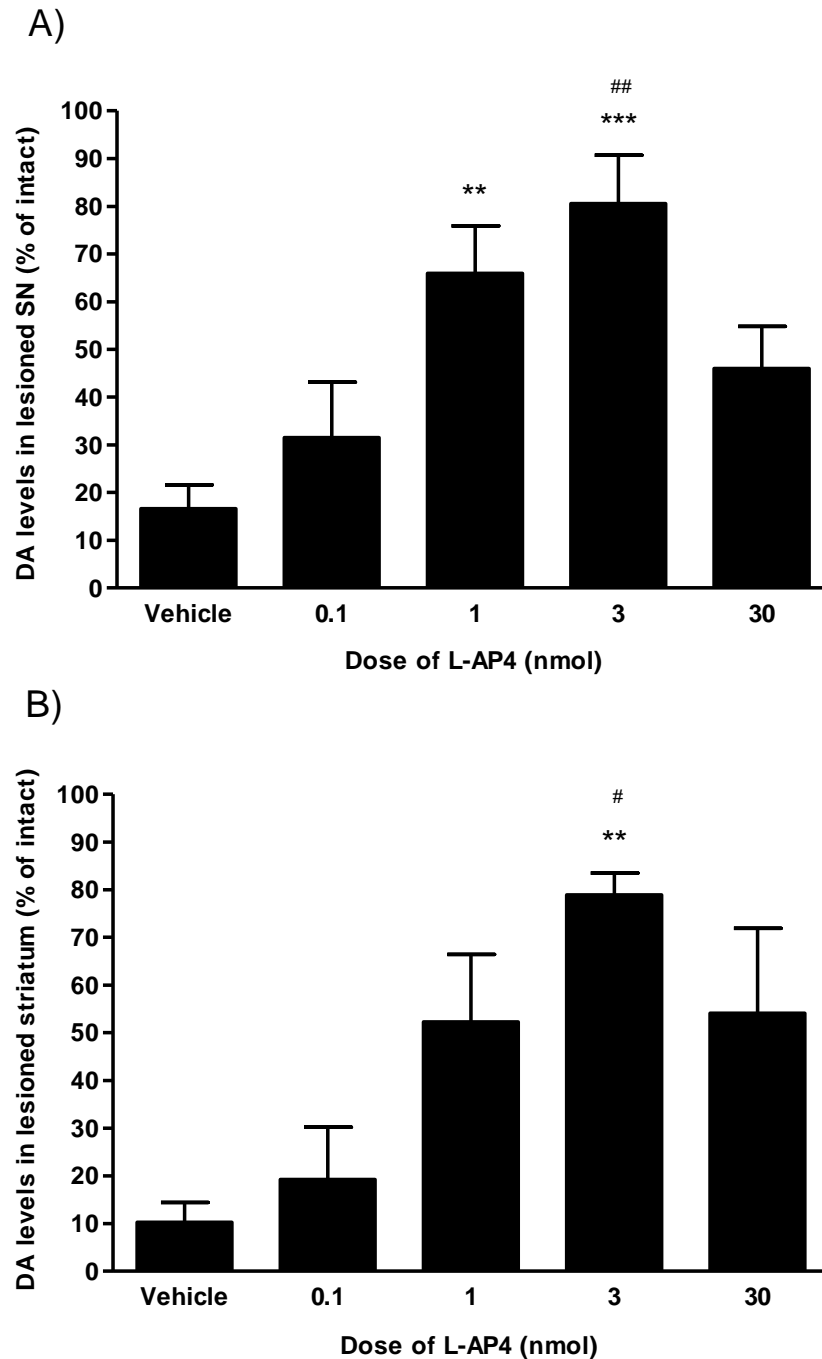
**Figure 3.9. (A)** The effects of sub-chronic supranigral infusion of L-AP4 (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of DDC in the striatum on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=7 for 1 and 3nmol L-AP4, n=8 for 30nmol L-AP4). \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 8.283; df = 3, 26. **(B)** Representative photomicrographs showing the levels of DDC immunoreactivity in striatal sections from 6-OHDA-lesioned animals following sub-chronic L-AP4 treatment. Fixed coronal sections (shown here + 0.20mm AP from bregma, Paxinos, 1998) were reacted with antibodies raised against DDC and stained with diaminobenzidine (DAB). Lesion side on the right; scale bar: 500 $\mu$ m.

#### 3.3.1.4 Effects of sub-chronic L-AP4 infusion on dopamine content in the substantia nigra and striatum

When assayed in a separate study using HPLC, nigral dopamine content was shown to be preserved in the lesioned SN of animals treated with L-AP4. In vehicle-treated 6-OHDA lesioned animals, nigral dopamine content fell from  $3629 \pm 912 \text{ ng g}^{-1}$  in the intact hemisphere to  $505 \pm 159 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $16.7 \pm 5.0\%$  ( $n=8$ ) of the intact hemisphere (figure 3.10A). L-AP4 treatment produced a dose-dependent preservation of nigral dopamine content, which reached a maximal significant effect at 3nmol L-AP4, where dopamine content in the lesioned hemisphere equated to  $80.5 \pm 10.2\%$  of the intact hemisphere ( $P < 0.001$  versus vehicle treatment; 1-way ANOVA with Bonferroni's *post-hoc* test). At a higher dose of L-AP4 (30nmol), dopamine content was no longer significantly preserved in the lesioned SN.

Effects on striatal dopamine content mirrored these seen in the SN. Thus, striatal dopamine content was also shown to be preserved in the lesioned hemisphere of animals treated with L-AP4. In vehicle-treated 6-OHDA lesioned animals, striatal dopamine content fell from  $13450 \pm 1351 \text{ ng g}^{-1}$  in the intact hemisphere to  $1352 \pm 560 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $10.3 \pm 4.1\%$  ( $n=8$ ) of the intact hemisphere (figure 3.10B). In tune with nigral dopamine content, L-AP4 treatment produced a dose-dependent preservation of striatal dopamine content, reaching a maximal significance effect with 3nmol L-AP4 ( $P < 0.01$  versus vehicle treatment; 1-way ANOVA with Bonferroni's *post-hoc* test), where dopamine content in the lesioned hemisphere remained at  $78.9 \pm 4.1\%$  of the intact hemisphere. At a higher dose of L-AP4 (30nmol), preservation of striatal dopamine content was reduced with respect to 3nmol L-AP4 yet remained significantly preserved ( $P < 0.05$  versus vehicle-treated animals; 1-way ANOVA with Bonferroni's *post-hoc* test).

Figure 3.10: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on dopamine content in the substantia nigra and striatum of unilaterally 6-OHDA lesioned rats.



**Figure 3.10.** The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol in 4µl) or vehicle (4µl PBS) on the 6-OHDA induced loss of dopamine content in the SN (**A**) and striatum (**B**) on the lesioned side expressed as a percentage of the intact side. For both, values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=6 for 0.1nmol, n=7 for 1, 3 and 30nmol L-AP4). \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). # (P < 0.05) and ## (P < 0.01) indicates significance difference compared to L-AP4 (0.1nmol) treated animals. In (**A**) F = 8.345; df = 4,30, (**B**) F = 6.194; df = 4,30.

### **3.3.1.5 Effects of sub-chronic L-AP4 infusion on motor behaviour in 6-OHDA lesioned rats**

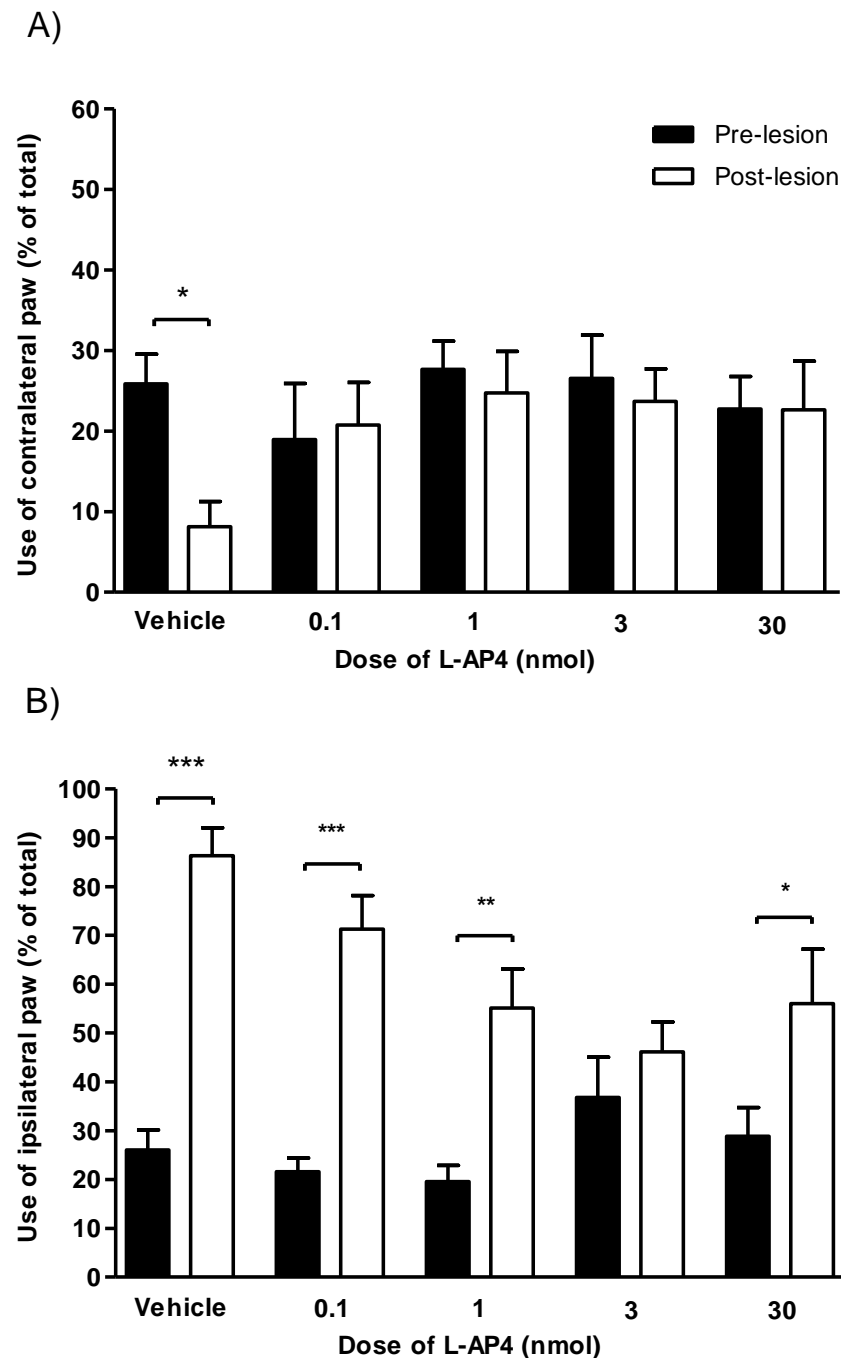
#### *Cylinder Test*

The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats were assessed using the cylinder test on day 5 post lesion. Ipsilateral, contralateral or use of both paws were quantified as a % of total paw use, where naive non-lesioned animals demonstrate preference to use both paws (~60%) ahead of ipsilateral (~20%) and contralateral (~20%) paws. Since a unilateral 6-OHDA lesion typically induces a functional deficit in contralateral paw, use of this paw is illustrated in figure 3.11A. As a result of impaired contralateral paw use it is common to observe compensatory increases in ipsilateral paw use shown in figure 3.11B.

As expected in vehicle treated animals, there was a significant decrease in use of the contralateral paw from  $25.9 \pm 3.7\%$  pre lesion to  $8.1 \pm 3.1\%$  post lesion confirming that a unilateral 6-OHDA lesion induced a reduction of approximately 69% in contralateral paw use (figure 3.11A). In contrast, in animals treated with 0.1, 3 and 30nmol L-AP4 use of contralateral paw was largely spared following a 6-OHDA lesion. For example, following treatment with L-AP4 (3 nmol) contralateral paw use equated to  $23.7 \pm 4.0\%$  of total paw use post-lesion in comparison to  $26.6 \pm 5.4\%$  pre-lesion, demonstrating approximately 89% preservation or 11% reduction in contralateral paw use post-lesion following L-AP4 treatment.

Interestingly a marked increase in ipsilateral paw use was observed in vehicle treated animals following a 6-OHDA lesion in tune with the significant decrease in contralateral paw use. However with increasing doses of L-AP4 this compensatory increase in use of ipsilateral paw decreased as use of the contralateral paw was preserved following L-AP4 treatment. For example in vehicle treated animals, ipsilateral paw use post lesion increased by >230% with respect to pre-lesion score, compensating for an impaired contralateral paw. However in L-AP4 treated animals (3nmol), ipsilateral paw use post lesion increased by only 25% of pre-lesion score confirming the reduced asymmetry in these animals (figure 3.11B).

Figure 3.11: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



**Figure 3.11.** The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol in 4  $\mu$ l) or vehicle (4 $\mu$ l PBS) on contralateral (A) and ipsilateral (B) paw use expressed as a percentage of total paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion. For all, values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=6 for 0.1nmol, n=7 for 1, 3 and 30nmol L-AP4). \* (P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for pre/post lesion factor, F = 2.135, df = 1,60; for treatment factor F = 1.538, df = 4,60. In (B) for pre/post lesion factor, F = 73.49, df = 1,60; for treatment factor F = 2.478, df = 4,60.

### *Adjusted Stepping*

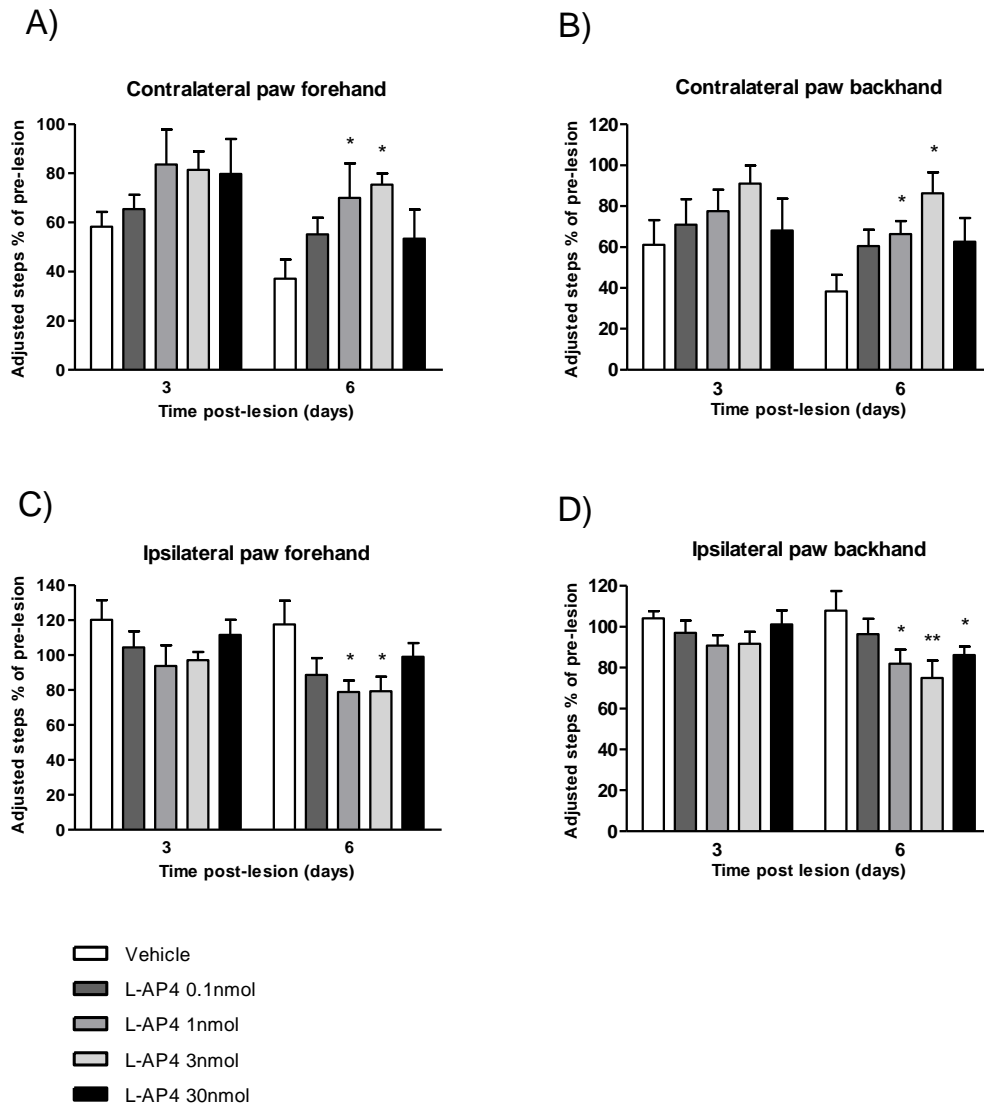
The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 3.12. As predicted the contralateral paw (lesioned) demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $58.3 \pm 6.1\%$  at 3 days post-lesion and  $37.0 \pm 7.8\%$  at 6 days in the vehicle treated group, indicating unilateral 6-OHDA lesioning reduced the number of adjusted steps in this direction (figure 3.12A). Infusion with L-AP4 protected against the decline in contralateral paw use, again displaying a bell-shaped dose response profile. For example, use of the contralateral paw increased in a dose dependent manner reaching maximal effect with L-AP4 (3nmol) equating to  $81.4 \pm 7.5\%$  of pre-lesion scores at 3 days post-lesion and  $75.4 \pm 4.6\%$  at 6 days, the latter of which was a significant effect with respect to vehicle-treated animals ( $P < 0.05$ ). However, with a high dose of L-AP4 (30nmol), preservation of contralateral paw use was markedly reduced from  $79.7 \pm 14.2\%$  of pre-lesion score at 3 days post-lesion and  $53.3 \pm 11.9\%$  at 6 days.

In the backhand direction, there was also a marked reduction in contralateral paw use in vehicle treated animals. This amounted to a decrease in adjusted steps from 100% pre-lesion to  $61.2 \pm 12.1\%$  at 3 days post-lesion and  $38.3 \pm 8.1\%$  at 6 days confirming unilateral 6-OHDA-lesioning also reduced the number of adjusted steps in the backhand direction (figure 3.12B). However following L-AP4 treatment use of the contralateral paw in the backhand direction was significantly preserved, again reaching maximal effect with the 3nmol dose whereby  $91.1 \pm 8.8\%$  of pre-lesion scores at 3 days and  $86.2 \pm 10.2\%$  at 6 days were obtained. In the high-dose L-AP4 (30nmol) group there was no significant increase in adjusted steps compared to vehicle group consistent with the bell-shaped dose response profile seen in the forehand direction. Collectively these findings demonstrate supranigral sub-chronic L-AP4 (3nmol) treatment reduced the motor asymmetry induced by a unilateral 6-OHDA lesion shown here by increasing the contralateral adjusted stepping in both forehand and backhand directions, almost restoring contralateral paw use to pre-lesion levels.

As expected the ipsilateral paw (non-lesioned) in both the forehand and backhand direction showed no significant deficit in adjusted steps 3 or 6 days post-lesion in vehicle treated animals, although there was a tendency for a reduction in adjusted steps in the L-AP4 treated groups (figure 3.12C and D). Indeed this effect is similar to that seen on assessment of spontaneous motor function in the cylinder test and also likely reflects the reduced asymmetry in L-AP4 treated animals.



Figure 3.12: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on motor function assessed using the adjusted stepping test in unilaterally 6-OHDA lesioned rats.



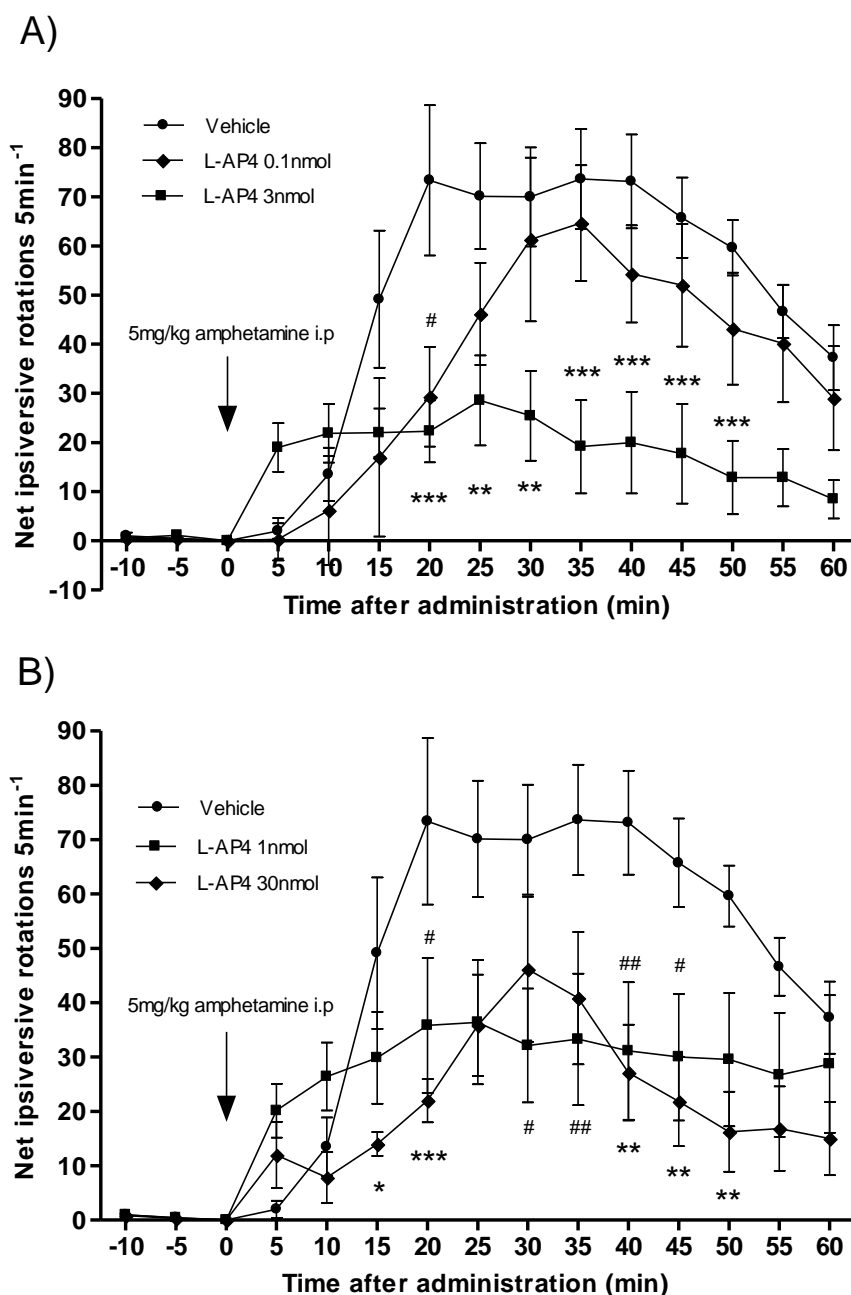
**Figure 3.12.** The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol in 4µl) or vehicle (4µl PBS) on contralateral and ipsilateral paw use in the forehand and backhand direction (A-D) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion expressed as a percentage of pre-lesion score. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=6 for 0.1nmol, n=7 for 1, 3 and 30nmol L-AP4). \*(P < 0.05) and \*\* (P < 0.01) indicates significant differences compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for treatment factor, F = 3.382, df = 4,60 and for days post lesion factor, F = 5.897, df = 1,60. In (B) treatment factor, F = 3.65, df = 4,60 and days post lesion factor, F = 2.613, df = 1,60. In (C) treatment factor, F = 4.084, df = 4,60 and for days post lesion factor, F = 4.275, df = 1,60). In (D) treatment factor, F = 3.806, df = 4,60 and days post lesion factor, F = 3.103, df = 1,60).

### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic L-AP4 or vehicle are shown in figure 3.13. Both graphs A and B show the same vehicle group split with two doses of L-AP4 for clarity. Analysis of time-course in vehicle treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. However in animals treated with L-AP4 a significant reduction in amphetamine-induced ipsiversive rotations was noted. L-AP4 (3nmol) treated animals demonstrated the greatest reduction in ipsiversive rotations reaching a significant difference over a period of 35 min from 20 to 55 min ( $P < 0.001-0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test) compared to vehicle-treated animals (figure 3.13A). Furthermore, figure 3.13B demonstrated L-AP4 (30nmol) also induced a significant reduction in ipsiversive rotations at numerous intervals over a 60 min time period ( $P < 0.001-0.05$ , 2 way ANOVA with Bonferroni's *post-hoc* test) compared to vehicle-treated animals.

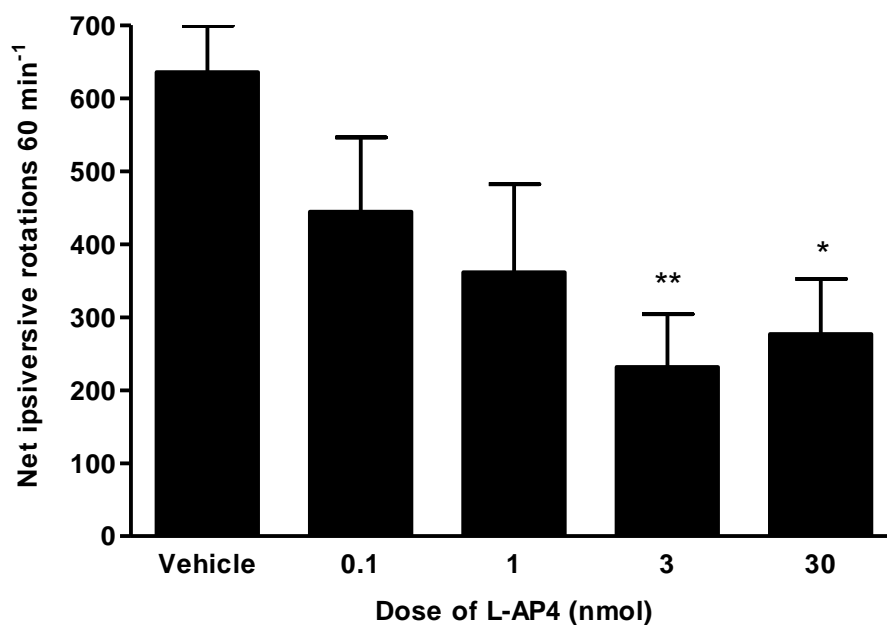
In figure 3.14, the mean total ipsiversive rotations were quantified over the 60 min period. The number of ipsiversive rotations by amphetamine ( $5\text{mg.kg}^{-1}$ ) in vehicle treated animals was  $636 \pm 64$  60 rotations in  $60\text{ min}^{-1}$ . L-AP4 dose dependently decreased amphetamine-induced ipsiversive rotations reaching maximal effect with L-AP4 (3nmol) where the total mean number of rotations equated to  $232 \pm 72$ , demonstrating a 64% reduction compared to vehicle treated animals ( $P < 0.01$ , 1-way ANOVA with Dunnett's *post-hoc* test). A higher dose of L-AP4 (30nmol) also significantly reduced the number of rotations to  $277 \pm 75$  rotations in  $60\text{ min}^{-1}$  ( $P < 0.05$ ) however to a lesser extent than L-AP4 (3nmol) consistent with a bell-shaped dose response profile reported in previous behavioural tests. Collectively, these findings demonstrate L-AP4 mediated functional neuroprotection by effectively reducing motor asymmetry induced by unilateral 6-OHDA-lesioning.

Figure 3.13: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 3.13.** The effects of sub-chronic supranigral infusion of L-AP4 (0.1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. **(A)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle (1x PBS) and L-AP4 (0.1, 3 nmol) treated animals. **(B)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle (1x PBS) and L-AP4 (1,30nmol) treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=6 for 0.1nmol, n=7 for 1, 3 and 30nmol L-AP4). **(A)** \* (P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences between L-AP4 (3 and 30nmol) and vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). **(B)** # (P < 0.05) indicates significant difference between L-AP4 (0.1 and 1nmol) and vehicle treated animals. Treatment factor, F = 25.05, df = 4,450 and for time, F = 19.33, df = 14,450.

Figure 3.14: Effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor agonist L-AP4, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 3.14.** Total ipsiversive rotations induced by amphetamine in 60 min<sup>-1</sup> in vehicle and L-AP4 treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, n=6 for 0.1nmol, n=7 for 1, 3 and 30nmol L-AP4). \* (P <0.05), \*\* (P <0.01) indicates a significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 3.595; df = 4, 30.

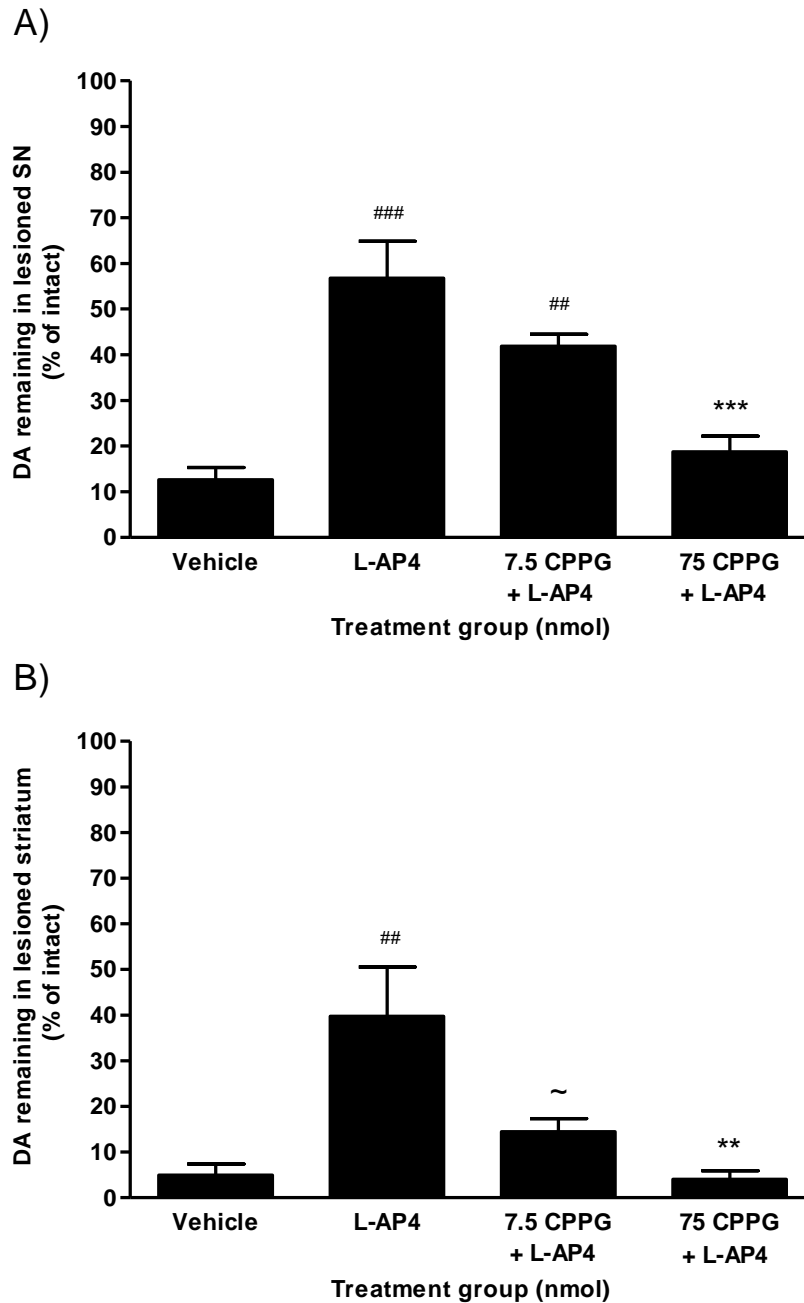
### **3.3.2 Effects of pre-treatment with group III mGlu receptor antagonist CPPG, on L-AP4 mediated neuroprotection in unilaterally 6-OHDA-lesioned rats**

#### **3.3.2.1 Effects of pre-treatment with CPPG on L-AP4 mediated neuroprotection with respect to dopamine content in the substantia nigra and striatum**

In a separate study, the protective effects of L-AP4 (3nmol) in preserving dopamine content in the SN and striatum following a unilateral 6-OHDA lesion were found to be significantly inhibited by pre-treatment with CPPG (75nmol). In the lesioned hemisphere preservation of nigral dopamine following L-AP4 treatment significantly fell from  $56.8 \pm 8.1\%$  of the intact hemisphere to  $18.8 \pm 3.4\%$  following pre-treatment with CPPG (75nmol) ( $P < 0.001$ , 1-way ANOVA with Bonferroni's *post-hoc* test). Pre-treatment with a low dose of CPPG (7.5nmol) failed to significantly inhibit preservation mediated by L-AP4 where  $41.9 \pm 2.6\%$  of nigral dopamine remained in the lesioned hemisphere (figure 3.15A).

L-AP4 mediated preservation of striatal dopamine was also significantly inhibited following pre-treatment with CPPG (75nmol) where dopamine content fell from  $39.8 \pm 10.8\%$  of the intact hemisphere to  $4.1 \pm 1.9\%$  following pre-treatment with CPPG ( $P < 0.01$ , 1-way ANOVA with Bonferroni's *post-hoc* test). Pre-treatment with the low dose of CPPG (7.5nmol) also significantly inhibited preservation of striatal dopamine mediated by L-AP4 ( $P < 0.05$ ), although to a lesser extent where  $14.4 \pm 2.9\%$  of striatal dopamine content remained in the lesioned hemisphere (figure 3.15B).

Figure 3.15: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of L-AP4, with respect to dopamine content in the substantia nigra and striatum of unilaterally 6-OHDA lesioned rats.



**Figure 3.15.** The effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor antagonist CPPG (7.5 or 75nmol in 4 $\mu$ l of 0.1M NaOH) on the protective effect of L-AP4 (3nmol in 4 $\mu$ l) proceeding 6-OHDA induced loss of dopamine content in (A) the substantia nigra (SNpc) and (B) striatum. Values represent mean  $\pm$  s.e.m. (n=6 for vehicle, n=8 for 7.5nmol CPPG, n=7 for 75nmol CPPG and 3nmol L-AP4). \*\* (P<0.01) and \*\*\* (P<0.001) indicate a significant difference compared to L-AP4 (3nmol) treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). ## (P<0.01) and ### (P<0.001) indicates a significant difference compared to vehicle-treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). ~ (P<0.05) indicates a significant difference compared to L-AP4 (3nmol) treated animals (1-way ANOVA with Bonferroni's *post hoc* test). For (A) F = 17.55; df = 3,24, (B) F = 8.035; df = 3,24.

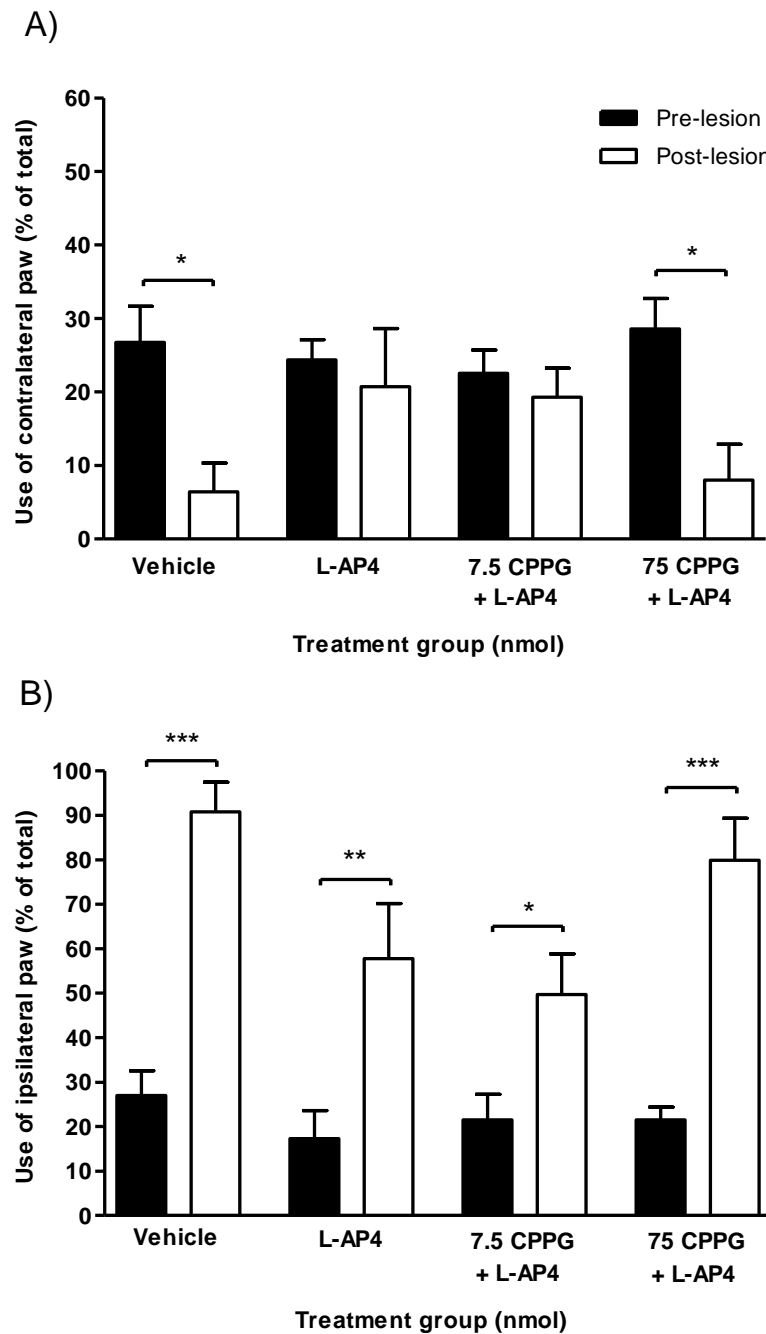
#### **4.3.2.2 Effects of pre-treatment with CPPG on L-AP4 mediated neuroprotection with respect to motor function in 6-OHDA lesioned rats**

##### *Cylinder Test*

The effects of CPPG pre-treatment with L-AP4 (3nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats was assessed using the cylinder test on day 5 post lesion (figure 3.16). As expected in vehicle treated animals, there was a significant decrease in use of the contralateral paw from  $26.8 \pm 4.9\%$  pre-lesion to  $6.4 \pm 4.0\%$  post lesion. Consistent with the previous study in this chapter, use of the contralateral paw was largely spared with L-AP4 (3nmol) following a 6-OHDA lesion. Contralateral paw use equated to  $20.8 \pm 7.9\%$  of total paw use post-lesion in comparison to  $24.4 \pm 2.8\%$  pre-lesion, demonstrating approximately 85% preservation in contralateral paw use post-lesion following L-AP4 treatment which is in good agreement with our first study in this chapter (89% preservation). Pre-treatment with CPPG (75nmol) almost totally inhibited preservation of contralateral paw use with L-AP4 from  $28.6 \pm 4.1\%$  pre-lesion to  $8.0 \pm 4.9\%$  post-lesion, attaining a similar post-lesion score to that of vehicle treated animals ( $6.4 \pm 4.0\%$  post-lesion, figure 3.16A). The lower dose of CPPG (7.5nmol) failed to block L-AP4's actions.

In the previous study, as a result of preserving contralateral paw use, L-AP4 decreased the compensatory increase in ipsilateral paw use as described in vehicle-treated animals. Indeed, in this study, ipsilateral paw use in vehicle-treated animals post-lesion increased by >236% with respect to pre-lesion score, compensating for an impaired contralateral paw. Interestingly in L-AP4 (3nmol) treated animals, the ipsilateral paw use post lesion also increased by a similar extent (233% of pre-lesion score). However it is likely this is due to a lower pre-lesion score being obtained here of  $17.34 \pm 6.3\%$  in comparison to  $36.8 \pm 8.3\%$  in the previous study. Nevertheless, in CPPG (75nmol) pre-treated animals, use of the ipsilateral paw post-lesion increased by 271% (figure 3.16B) confirming impaired motor function in these animals, consistent with the notion CPPG acts to inhibit functional neuroprotection mediated by L-AP4.

Figure 3.16: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of L-AP4 with respect to motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



**Figure 3.16.** The effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor antagonist CPPG (7.5 or 75nmol in 4 $\mu$ l of NaOH) on the protective effect of L-AP4 (3nmol in 4 $\mu$ l), with respect to (A) contralateral and (B) ipsilateral paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion expressed as a percentage of total paw use. For all, values represent mean  $\pm$  s.e.m. (n=6 for vehicle, n=8 for 7.5nmol CPPG, n=7 for 75nmol CPPG and L-AP4). \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for pre/post lesion factor,  $F = 13.03$ ,  $df = 1,48$ ; for treatment factor,  $F = 0.6224$ ,  $df = 3,48$ . In (B) for pre/post lesion factor,  $F = 72.35$ ,  $df = 1,48$  and for treatment factor  $F = 3.795$ ,  $df = 3,48$ .

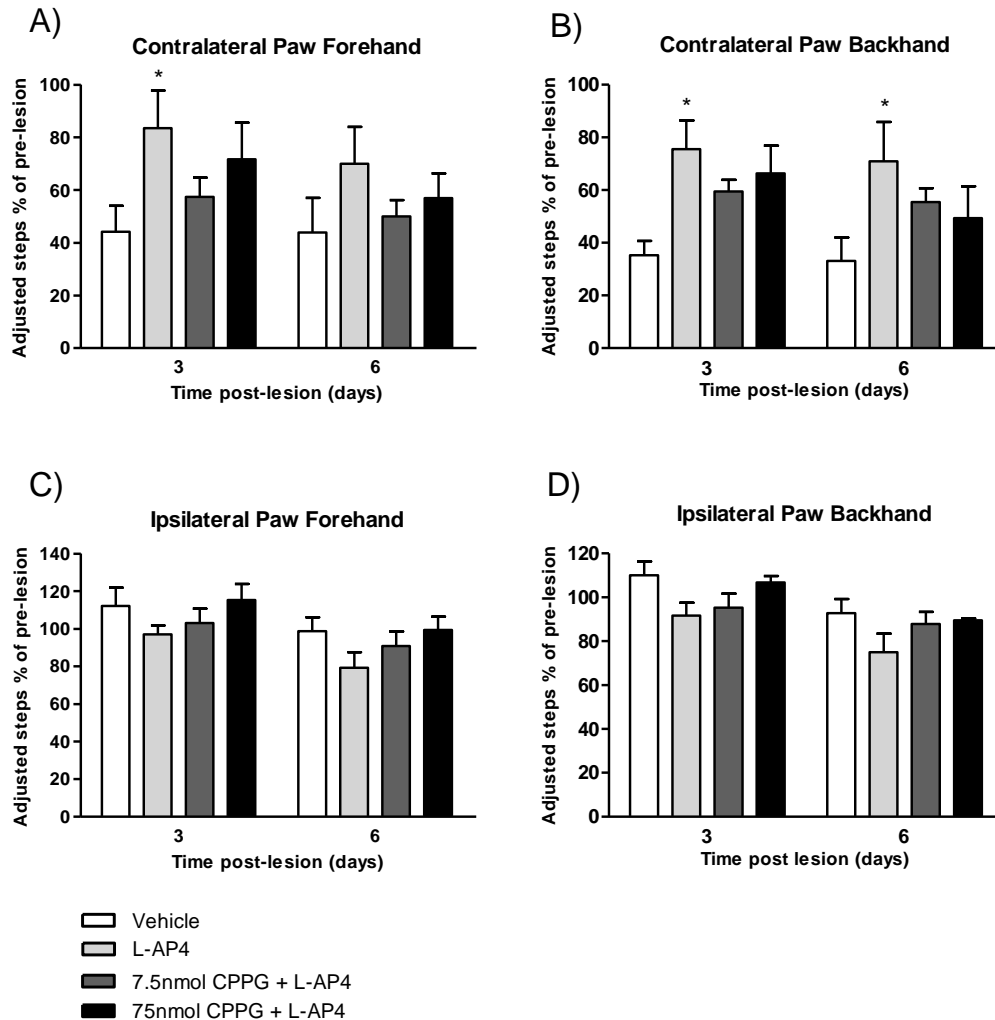


### *Adjusted Stepping*

The effects of CPPG pre-treatment with L-AP4 (3nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 3.17. As predicted, the contralateral paw (lesioned) demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $43.9 \pm 13.1\%$  at 6 days post-lesion in the vehicle treated group, indicating unilateral 6-OHDA lesioning reduced the number of adjusted steps in this direction (figure 3.17A). In this study, L-AP4 (3nmol) treatment preserved contralateral paw use, equating to  $70.0 \pm 14.0\%$  of pre-lesion scores at 6 days post-lesion, with respect to vehicle-treated animals. Following pre-treatment with CPPG however, this preservation was reduced to  $57.0 \pm 9.4\%$  of pre-lesion scores at 6 days post-lesion. A similar effect following CPPG pre-treatment was also observed in the backhand direction. L-AP4 (3nmol) treatment significantly preserved contralateral paw use, equating to  $70.9 \pm 15.0\%$  of pre-lesion scores at 6 days post-lesion, with respect to vehicle-treated animals ( $P < 0.05$ ). However, following pre-treatment with a high dose of CPPG, preservation was reduced, on this occasion to  $49.3 \pm 12.2\%$  of pre-lesion scores at 6 days post-lesion (figure 3.17B). Since the effects of CPPG were not significant here, this may suggest the optimal dose of antagonist may not have been reached.

As expected the ipsilateral paw (non-lesioned) in the forehand direction showed no significant deficit in adjusted steps 3 or 6 days post-lesion in vehicle treated animals, although a tendency for an L-AP4 mediated reduction in adjusted steps as previously reported was also seen here in both directions (figure 3.17 C,D). This tendency was not apparent following pre-treatment with CPPG (75nmol) where scores were similar to those obtained to vehicle treated animals. Collectively, these findings demonstrate pre-treatment with CPPG inhibited L-AP4 mediated preservation of motor function shown here by reducing contralateral paw use.

Figure 3.17: Effects of the III mGlu receptor antagonist CPPG on the protective effect of L-AP4, with respect to motor function assessed using the adjusted steps test in unilaterally 6-OHDA lesioned rats.



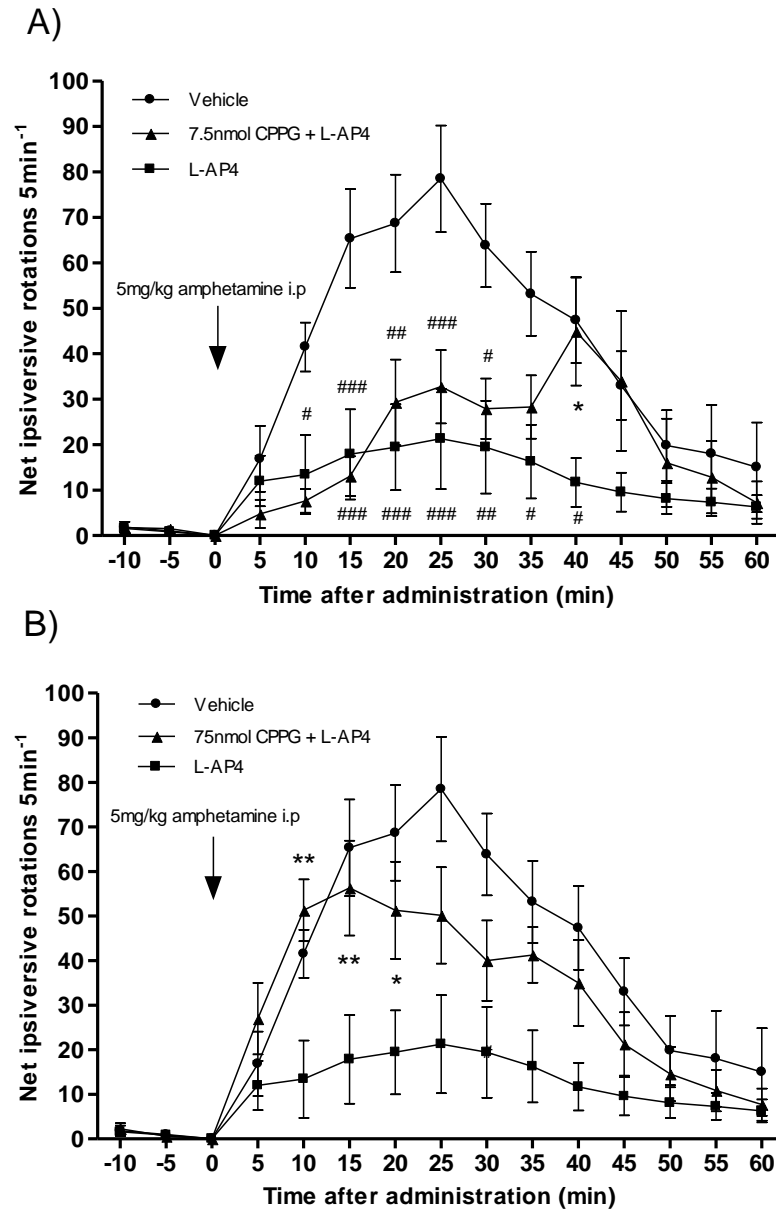
**Figure 3.17.** The effects of the group III mGlu receptor antagonist CPPG (7.5 or 75nmol in 4μl of 0.1M NaOH) on the protective effect of L-AP4 (3nmol in 4μl) or vehicle (4μl 0.1M NaOH) with respect to contralateral and ipsilateral paw use in the forehand and backhand direction (**A-D**) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion expressed as a percentage of pre-lesion score. Values represent mean  $\pm$  s.e.m. (n=6 for vehicle, n=8 for 7.5nmol CPPG, n=7 for 75nmol CPPG and L-AP4). \* ( $P < 0.05$ ) indicates a significant difference compared to vehicle-treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). For (**A**), treatment factor  $F = 3.018$ ,  $df = 3,48$  and for days post-lesion factor,  $F = 1.288$ ,  $df = 1,48$ . For (**B**), treatment factor  $F = 5.074$ ,  $df = 3,48$  and for days post-lesion factor,  $F = 1.036$ ,  $df = 1,48$ . For (**C**), treatment factor  $F = 2.518$ ,  $df = 3,48$  and for days post-lesion factor,  $F = 7.232$ ,  $df = 1,48$ . For (**D**), treatment factor  $F = 3.605$ ,  $df = 3,48$  and for days post-lesion factor,  $F = 12.52$ ,  $df = 1,48$ .

### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic L-AP4 following pre-treatment with CPPG are shown in figure 3.18. Analysis of time-course in vehicle treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. As expected in animals treated with L-AP4 (3nmol) a significant reduction in amphetamine-induced ipsiversive rotations were identified, seen here over a period of 25 min from 15 to 40 min ( $P < 0.001-0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test) compared to vehicle-treated animals (figure 3.18A). Pre-treatment with a low dose of CPPG failed to inhibit these effects of L-AP4 where a significant inhibition of ipsiversive rotations was reported at one time-point only (40 min) following amphetamine ( $P < 0.05$ , 2 way ANOVA with Bonferroni's *post-hoc* test) compared to L-AP4 treated animals (figure 3.18A). However, pre-treatment with a high dose of CPPG (75nmol) revealed considerable inhibition of L-AP4 mediated reduction in ipsiversive rotations, reaching significance between 10 to 20 min ( $P < 0.01-0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test, figure 3.18B).

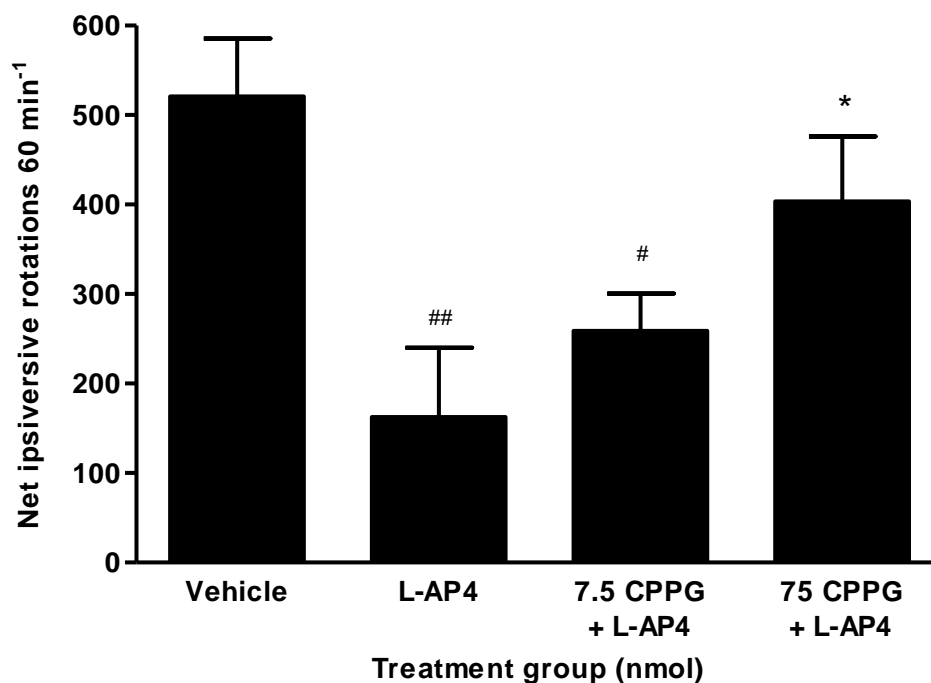
In figure 3.19, the mean total ipsiversive rotations were quantified over the 60 min period to identify the effects of CPPG on L-AP4 mediated reduction in ipsiversive rotations. L-AP4 (3nmol), as expected, significantly decreased amphetamine-induced ipsiversive rotations by up to 69% in comparison to vehicle treated animals ( $P < 0.01$ , 1-way ANOVA with Dunnett's *post-hoc* test). Pre-treatment with CPPG (75nmol), however, significantly inhibited L-AP4 mediated reduction in amphetamine-induced rotations. Using this high dose of CPPG the number of rotations equated to  $404 \pm 73$  in  $60\text{ min}^{-1}$  in comparison to  $163 \pm 77$  in L-AP4 treated animals ( $P < 0.05$ , 1-way ANOVA with Dunnett's *post-hoc* test). CPPG (7.5nmol) also inhibited the L-AP4 mediated reduction in ipsiversive rotations, where the number of rotations equated to  $259 \pm 42$   $60\text{ min}^{-1}$ , although this effect was not significant. Indeed this response was significantly different to vehicle treated animals, suggesting L-AP4 mediated reduction in rotations predominates at this dose of CPPG.

Figure 3.18: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of L-AP4, with respect to motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 3.18.** Effects of the group III mGlu receptor antagonist CPPG (7.5 or 75nmol in 4 $\mu$ l of 0.1M NaOH) on the protective effect of L-AP4 (3nmol in 4 $\mu$ l 1x PBS), with respect to amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. **(A)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle (0.1M NaOH), L-AP4 (3 nmol) and 7.5nmol CPPG pre-treated animals. **(B)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle, L-AP4 (3nmol) and 75nmol CPPG pre-treated animals. Values represent mean  $\pm$  s.e.m. (n=6 for vehicle, n=8 for 7.5nmol CPPG, n=7 for 3nmol L-AP4 and 75nmol CPPG). **(A)** \* (P <0.05) indicates significant difference between L-AP4 and 7.5nmol CPPG pre-treated animals. # (P <0.05), ## (P <0.01) and ### (P <0.001) indicate significant difference compared to vehicle treated animals. For treatment factor, F = 37.26, df = 2,270 and for time, F = 11.80, df = 14, 270. **(B)** \* (P <0.05) and \*\* (P <0.01) indicate significant difference between L-AP4 and 75nmol CPPG pre-treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). Treatment factor, F = 40.80, df = 2,255 and for time, F = 17.27, df = 14, 270.

Figure 3.19: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of L-AP4, with respect to motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 3.19.** Total ipsiversive rotations induced by amphetamine in 60 min<sup>-1</sup> in vehicle, L-AP4 and CPPG pre-treated animals. Values represent mean  $\pm$  s.e.m. (n=6 for vehicle, n=8 for 7.5nmol CPPG, n=7 for 75nmol CPPG and L-AP4). \* (P < 0.05) indicates a significant difference compared to 3nmol L-AP4 treated animals (1-way ANOVA with Dunnett's *post-hoc* test). # (P < 0.05), ## (P < 0.01) indicates significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 5.662; df = 3,24.

### 3.4 Discussion

These studies set out to examine the neuroprotective potential of L-AP4 in a rodent model of PD bearing a 6-OHDA lesion. On the basis of producing a sufficiently large lesion to offer the maximum potential for protection, as well as the greatest range to detect motor behavioural improvements, it was decided a deficit of 80-90% ‘full lesion’ would be required for these studies. The dose of 6-OHDA required to achieve this lesion size varies widely amongst different laboratories. For example the study of Vernon *et al.*, (2005) found an intranigral administration of 12µg of 6-OHDA produced a lesion size of 65%, according to loss of TH positive cells in the SNpc 8 days post-lesion. However a similar study found that 4µg 6-OHDA resulted in an 85-90% loss of nigral TH positive cells (O'Neill *et al.*, 2004). The discrepancies in lesion size are likely a result of different stereotactic coordinates for the SNpc, different infusion rates, or even different formulations and batches of 6-OHDA, which is a very unstable compound. In our hands 12µg of 6-OHDA was required to produce the desired ‘full lesion’ where 83% and 87% loss of nigral dopamine content was obtained in vehicle treated animals in studies described in section 3.3.1 and section 3.3.2 respectively, demonstrating a fairly reproducible lesion. With this in mind and to maintain consistency, 12µg of 6-OHDA was used in all subsequent studies described in this thesis.

Analysis of striatal TH in individual quadrants following a 6-OHDA lesion in the SNpc revealed there was a slight tendency for greater loss of TH staining in the dorsal striatum following a 6-OHDA lesion with up to 96% loss in the dorsal lateral region, compared to an 80% loss in the ventral medial striatum. This pattern of TH gradient loss albeit much more exaggerated has been previously reported by O'Neill *et al.*, (2004) who revealed an 80-90% loss of TH staining in the dorsal striatum, whilst seeing only a 50-60% loss in the ventral striatum. In the present study however the TH loss between quadrants in all treatment groups was not significantly different, therefore for all further studies described in this thesis, the mean total striatal TH was presented. It is important to emphasise at this point, using a ‘full lesion’ model as described here, in which 6-OHDA is injected directly into the SNpc, cell death is rapid. Under this paradigm, the majority of cell death occurs within the first 3d of

injection (Zuch *et al.*, 2000) and for this reason neuroprotective drug interventions are often, as in this case, introduced just prior to toxin injection. Such a design does not of course reflect the timing of drug interventions in the clinic and future studies should aim to reinforce these findings in a slower retrograde lesion model in which there is a sufficient time window to delay treatment.

Accepting this caveat, these studies revealed that L-AP4, administered 1h before and for 7 days after 6-OHDA injection protected against a nigrostriatal tract lesion. This was evidenced by a preservation of TH-positive cell numbers in the SNpc, preservation of the dopaminergic marker dopa decarboxylase (DDC) in the striatum, as well as a reduction in 6-OHDA-induced loss of striatal TH immunoreactivity and dopamine levels. These effects were maximal at 3 nmol L-AP4 and, in the case of TH-positive cell counts and striatal dopamine content, were lost at the highest doses tested (L-AP4 30nmol). This bell-shaped dose response relationship is in keeping with the findings of Vernon *et al.*, (2007) who found maximal histological and neurochemical protection against a 6-OHDA lesion with 10 nmol L-AP4 and a similar loss of effect with a higher dose of L-AP4 (50nmol). Furthermore this bell-shaped response to L-AP4 has been noted in our laboratory for L-AP4 decreased D-aspartate release in nigral tissue prisms (Austin *et al.*, 2010) and in other electrophysiological studies (Valenti *et al.*, 2005) where the inhibitory effects of L-AP4 were lost at higher concentrations.

Numerous explanations have been suggested to underlie this loss of efficacy at higher concentrations. Firstly, loss of efficacy at higher doses of L-AP4 may be caused by activation of known mGlu7 receptors residing on GABAergic terminals leading to NMDA-induced toxicity. At higher concentrations of L-AP4, activation of mGlu7 receptors may cause an inhibition of GABA release, consequently removing the hyperpolarising influence of GABA on striatal neurones (Lafon-Cazal *et al.*, 1999b). However, one would have to question why this effect would predominate only at high doses of L-AP4. This could be explained by the relative potency of L-AP4 at the group III mGlu receptor subtypes, demonstrating a particularly low potency at mGlu7 receptors with respect to mGlu4/mGlu8 receptor subtypes ( $EC_{50}$  for L-AP4 at mGlu7 is 100x > than at mGlu4/8, values are shown in table 3.1). This would suggest

activation of mGlu7 receptors on GABAergic terminals would only occur at higher concentrations of agonist/L-AP4.

Such a bell-shaped concentration response profile may reflect a loss of pharmacological specificity at the higher doses, since L-AP4 is known to activate excitatory group I mGlu receptors with an  $EC_{50} > 1000 \mu M$  in comparison to between 0.2 and 100  $\mu M$  at the different group III mGlu receptors (Cartmell & Schoepp, 2000). However, as group I mGlu receptor activation has been shown to produce a similar functional inhibition of excitatory synaptic transmission in the SNpr at least (Wittmann *et al.*, 2001), this explanation seems unlikely. The most plausible explanation for this loss of efficacy at higher doses of L-AP4 may be a result of receptor desensitisation, as suggested to explain the similar bell-shaped nature of electrophysiological responses of L-AP4 at the striatopallidal synapse (Valenti *et al.*, 2003). Moreover, L-AP4 has been shown to cause rapid internalisation of at least two members of the group III mGlu receptors in cell lines, mGlu4 (at 100 $\mu M$ ; Iacovelli *et al.*, 2004) and mGlu7 (at 400 $\mu M$ ; Pelkey *et al.*, 2005), suggesting receptor desensitisation may be a likely contributing factor. Finally, our lab has demonstrated repeated administration of L-AP4, 4 hours after an intranigral dose, led to a 72% reduction in the reversal of akinesia in the reserpine treated rat (Broadstock, 2006, PhD Thesis). This suggests desensitisation of group III mGlu receptors may well be a consequence of repeated supranigral infusion of L-AP4 perhaps at the higher dose reached here. However, given the difficulty of predicting the exact concentration of agonist reaching the receptors following intracerebral dosing, such suggestions remain speculative at this stage and further studies are required to clarify if desensitisation is indeed the root cause for loss of efficacy at the high dose of L-AP4 tested here (30 nmol).

It is notable in this study that the protection afforded at the striatal level was greater and occurred at slightly lower doses than required to give significant protection at the nigral level. This outcome was not surprising as the toxin was introduced directly into the cell bodies so it may be harder to protect that area compared to the terminals. Indeed more robust protection is often seen in the 6-OHDA lesioned rats at the striatal level e.g. with AMPA potentiators (Murray *et al.*, 2003) and nicotinic agonists



(Visanji *et al.*, 2006). Alternatively, this discrepancy may reflect the different parameters being measured in these regions, i.e. cell numbers in the SNpc, versus density of terminal staining in the striatum. Given that there is huge compensation in the nigrostriatal system, it is possible that small changes at the level of the terminals can have effects on function, whereas the level of protection may need to be greater to see clear effects on TH-positive cell counts.

In addition to confirming the protective effects of L-AP4 at a neurochemical and histological level, this study demonstrated for the first time that the protection observed against a 6-OHDA lesion translated to a preservation of motor function. According to previous findings, the adjusted steps test requires a lesion of 50-60% to show any stepping deficit, with paw akinesia decreasing proportionally for lesion sizes 60-100% (Kirik *et al.*, 1998). Following sub-chronic treatment with L-AP4, a significant overall preservation of contralateral paw use was shown in adjusted steps, whereby 3nmol L-AP4 preserved paw use to ~75% of pre-lesion score in the forehand direction, at 6 days post lesion. To assess spontaneous motor function, the cylinder test revealed 3nmol L-AP4 almost totally preserved contralateral paw use equating to ~90% of pre-lesion scores. Motor function was also assessed using the gold standard amphetamine-induced rotational test whereby robust and proportional ipsiversive rotations have been reported for animals with lesion sizes between 50-100% (Kirik *et al.*, 1998; Moore *et al.*, 2001). Treatment with L-AP4 following a 6-OHDA lesion mediated a significant decrease (e.g. 64% with 3 nmol L-AP4) in amphetamine-induced rotations compared to vehicle treated animals. This data along with our neurochemical findings suggest there was an improvement in striatal dopamine release from the lesioned hemisphere acting to decrease dopamine asymmetry between the lesioned and intact hemispheres.

To confirm that the effects of L-AP4 were mediated by group III mGlu receptor activation, in a separate study we examined the ability of the broad spectrum antagonist, CPPG, to inhibit the response to L-AP4. CPPG is an orthosteric antagonist competing with endogenous glutamate for receptor binding, thus it was predicted to remove agonist binding required for group III mGlu receptor activation. Indeed we demonstrated here, daily pre-treatment with the selective group III mGlu receptor

antagonist, CPPG (75nmol), 30 min prior to L-AP4 administration abolished both neurochemical and functional protection mediated by our optimal dose of L-AP4 (3nmol). For example, preservation of nigral dopamine declined from 57% with respect to non-lesioned contralateral hemisphere in L-AP4 (3nmol) treated animals to 81% of non-lesioned hemisphere following pre-treatment with CPPG (75nmol). This is consistent with findings from Vernon et al who demonstrated L-AP4 (10nmol) responses were inhibited by the group III antagonist, M-SOP (50nmol) confirming a receptor mediated response (Vernon *et al.*, 2005).

Having reported L-AP4 mediated a profound neuroprotective effect in our 6-OHDA lesion model of PD, it is pertinent to suggest potential mechanisms underlying the protection described here. It is thought the neuroprotective nature of L-AP4 may be a consequence of presynaptic inhibition of glutamate from the STN, which is known to be overactive in the parkinsonian basal ganglia causing degeneration of dopaminergic neurons by glutamate-mediated excitotoxicity (Valenti *et al.*, 2005). Our laboratory has identified both *in vitro* using nigral slices and *in vivo* with microdialysis, L-AP4 inhibits glutamate release in the SNpr (Austin *et al.*, 2010). Although these studies did not focus on the SNpc, STN efferents arborise extensively so that they also innervate the SNpc and SNpr (Smith *et al.*, 1990). Thus it is conceivable that L-AP4 will serve to decrease glutamate release in the SNpc also. In support of this, electrophysiological studies have shown L-AP4 inhibits glutamate mediated excitatory post-synaptic potentials (EPSPs) in the SNpc (Katayama *et al.*, 2003; Wigmore & Lacey, 1998). The most plausible mechanism of neuroprotection by L-AP4 therefore, would be a reduction in glutamate-mediated excitotoxicity in SNpc neurones. However, there are several alternative mechanisms worthy of discussion here that may underlie neuroprotection mediated by group III mGlu receptor activation.

One such alternative may underlie the contribution of glia to mediate neuroprotection either by inhibition of pro-inflammatory cytokines or production of neurotrophic factors. Group III mGlu receptors are expressed by glial cells with both cerebellar astrocytes and microglia expressing mRNA for all group III mGlu receptors (Taylor *et al.*, 2003; Wroblewska *et al.*, 1998). Although an induction of neurotrophic factors

has not been demonstrated experimentally by activation of group III mGlu receptors, there is considerable evidence that activation of the similar  $G_{i/o}$  coupled group II mGlu receptors induce the release of neurotrophic factors. For example in mixed cortical cultures, the group II mGlu agonists have been shown to cause an induction of the neurotrophic factor TGF- $\beta$  in response to reactive astrocytes and microglia (Bruno *et al.*, 1998). In addition, group II mGlu receptor activation in 6-OHDA lesioned and MPP<sup>+</sup> treated mice has been shown to lead to a significant induction of BDNF and TGF- $\beta$  by reactive glia (Matarredona *et al.*, 2001; Murray *et al.*, 2002). This suggests a neurotrophic role for the closely related group III mGlu receptors may yet to be disclosed.

The anti-inflammatory role of group III mGlu receptor agonists is more widely reported. For example, in microglial conditioned medium or in mixed cultures, L-AP4 can reduce activation of microglia and subsequent neurotoxicity, following stimulation with an inflammatory insult such as LPS or chromogranin A (Taylor *et al.*, 2003). It is unclear at this point if this is due to an inhibition of neurotrophic factors or reduction in pro-inflammatory cytokine production. In support of the latter, group III mGlu receptor activation with L-AP4 has been demonstrated to decrease reactive gliosis in glial cultures, by inhibiting the chemokine, **Regulated upon Activation, Normal T-cell Expressed, and Secreted** (RANTES) (Besong *et al.*, 2002). RANTES is a pro-inflammatory cytokine, activating T cells, eosinophils and basophils. Therefore its inhibition by group III mGlu receptor activation may contribute to neuroprotection mediated by L-AP4, by inhibiting the neuroinflammatory response and reactive gliosis which occurs following cell death induced by 6-OHDA (as described in section 1.4.2.1). An additional glial-related mechanism may involve an increase in glutamate uptake by glial cells. L-AP4 has been shown to inhibit neurotoxicity induced by the complex I inhibitor MPP<sup>+</sup> restoring levels of glutamate uptake by cultured astrocytes to control levels thereby attenuating glutamate mediated excitotoxicity (Yao *et al.*, 2005). Furthermore the closely related group II mGlu receptors have been shown to produce an up-regulation of the glial glutamate transporters GLT-1 and GLAST through mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 (PI-3) kinase-dependent pathway (Aronica *et al.*, 2003). Since considerable microglial activation occurs in the 6-

OHDA lesion model of PD (Marinova-Mutafchieva *et al.*, 2009), it is conceivable that glial-mediated actions may contribute to the neuroprotection observed here with L-AP4.

L-AP4 mediated stimulation of group III mGlu receptors has been shown to stimulate MAP kinase and PI-3 kinase pathways in cultured cerebellar granule cells (Iacovelli *et al.*, 2004). These pathways are involved in numerous processes such as cell growth, proliferation and survival which may also lead to neuroprotective effects. In support of this, using specific inhibitors of these pathways, it was demonstrated these were necessary to provide neuroprotection against apoptosis induced by trophic deprivation (Iacovelli *et al.*, 2004). However, the exact mechanism in which these pathways mediate neuroprotection is still unknown.

Finally, a further mechanistic implication has derived from a study demonstrating L-AP4 could protect TH-positive neurones in rat embryonic midbrain cultures against the toxicity of rotenone (Jiang *et al.*, 2006). Rotenone is known to inhibit mitochondrial complex I and cause depolymerisation of microtubules, the latter of which is thought to be a key mechanism underlying rotenone's toxicity on dopaminergic neurones (Ren *et al.*, 2005). The protective effects of L-AP4 against rotenone toxicity are also thought to involve activation of the MAP kinase pathway, since L-AP4 increased activation of the MAP kinase extracellular signal-regulated kinase in midbrain TH-positive neurone cultures and protection against rotenone toxicity was blocked by pharmacological inhibition of MAP kinase kinase (Jiang *et al.*, 2006). Inhibition of MAP kinase kinase also prevented L-AP4-mediated attenuation of rotenone-induced microtubule depolymerisation, suggesting L-AP4 provided protection of TH-positive neurones through activation of the MAP kinase pathway to stabilize microtubules (Jiang *et al.*, 2006). Therefore, whilst neuroprotection with L-AP4 (3nmol), is considered to be largely mediated by a reduction of glutamate release from subthalamonigral terminals to prevent glutamate-mediated excitotoxicity, a role for glial cells must also be considered. Indeed, glial-mediated increases in glutamate uptake and inhibition of reactive gliosis, as well as activation of MAP kinase and PI3 kinase pathways may all be implicated in affording neuroprotective effects. The likely contribution from multiple mechanisms may

explain why protection with L-AP4 was maximal with a relatively low dose (3nmol) compared to that required to produce maximal reversal of reserpine-induced akinesia (300 nmol), which most probably relies on inhibition of glutamate release alone (Austin *et al.*, 2010). Although there is little in the way of changed expression of group III mGlu receptors following a 6-OHDA lesion (Messenger *et al.*, 2002), a post-lesion increase in sensitivity of the receptors may also explain why lower doses of L-AP4 were required in this model compared to the acute reserpine model.

In conclusion, the *in vivo* studies in this chapter confirm activation of group III mGlu receptors using the broad spectrum agonist L-AP4 mediated neuroprotection of the nigrostriatal tract in 6-OHDA lesioned rats. Furthermore these studies revealed for the first time, neuroprotection at the histological and neurochemical level translated to functional improvements in both forced and habitual behavioural tests indicating normalisation of dopamine asymmetry. In a separate study, pre-treatment with the broad spectrum antagonist CPPG significantly inhibited protection mediated by L-AP4 at the neurochemical and functional level confirming effects are group III mGlu receptor driven. Studies in the next two chapters seek to elucidate which group III mGlu receptors mediate neuroprotection by L-AP4. Recent findings would indicate mGlu4 as the lead candidate since the positive allosteric modulator (PAM) N-phenyl-7-(hydroxyimino)cyclopropa[b]-chromen-1a-carboximide (PHCCC) has been shown to reduce dopaminergic neuron degeneration in the MPTP model of PD (Battaglia *et al.*, 2006). However, no mGlu4 selective agents have yet been examined in the 6-OHDA lesion model of PD. Furthermore, the contribution of mGlu7 or mGlu8 in mediating functional neuroprotection has not yet been explored so a full pharmacological evaluation of the potential of these different group III mGlu receptors is warranted.

**Chapter 4:** Potential of targeting metabotropic glutamate receptor 4 to provide functional neuroprotection in the 6-OHDA rat model of PD

## 4.1 Introduction

In the previous chapter we demonstrated the broad spectrum group III mGlu receptor agonist L-AP4 (which activates mGlu4, 7 and 8 receptors), significantly protects against 6-OHDA-induced nigrostriatal tract degeneration in rats and mediates preservation of motor behaviour in these animals. To begin to elucidate the pharmacological identity of the group III mGlu receptor(s) mediating this protective effect, studies in this chapter sought to examine whether mGlu4 receptor activation alone could provide functional neuroprotection in the same experimental paradigm.

In Chapter 2 of this thesis, we demonstrated a particularly high intensity of immunoreactivity for mGlu4 in the SNpc. Furthermore electrophysiological studies have confirmed the function of these receptors, showing activation of mGlu4 using the positive allosteric modulator (PAM) PHCCC inhibits STN-evoked EPSCs in dopaminergic neurones of the SNpc (Valenti *et al.*, 2005). In addition, our lab have recently demonstrated activation of mGlu4 receptors in the SN using PHCCC (30 $\mu$ M), reduced glutamate release (shown by inhibition of [ $^3$ H]-D-aspartate release) from nigral slices *in vitro* (Broadstock *et al.*, 2011). Collectively, these findings propose activation of mGlu4 receptors in the SNpc could be predicted to inhibit glutamate-mediated excitotoxicity and thus provide protection against a 6-OHDA-induced lesion. In support of this notion, a previous report has demonstrated that a systemic injection of PHCCC (3mg kg $^{-1}$ ), given 30 min prior to toxin injection, provided approximately 50% protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of striatal dopamine content and reduction in TH-positive cell numbers in the SNpc in mice (Battaglia *et al.*, 2006). Moreover these neuroprotective effects of PHCCC were lost in mGlu4 knockout mice, confirming these effects were mGlu4-mediated. While these results with PHCCC are encouraging, the compound suffers from low potency (approximately 4  $\mu$ M *in vitro*), and poor aqueous solubility requiring DMSO which is known to be neurotoxic. Indeed in the study by Battaglia *et al.*, (2006) the vehicle for PHCCC, 50% DMSO injected alone led to an increase in MPTP toxicity. Furthermore, the pharmacology of PHCCC has also been questioned by demonstrating antagonism at the mGlu1 receptor at a potency similar to that of mGlu4 (Marino *et al.*, 2003). This property is

particularly undesired as antagonism of mGlu1 also leads to an inhibition of glutamate release, therefore any effects mediated by PHCCC likely implicate group I mGlu receptors limiting its utility as an mGlu4 selective PAM.

Accepting these shortcomings, the more recent mGlu4 PAM ( $\pm$ )-cis-2-(3,5-dichlorophenylcarbamoyl)cyclo-hexanecarboxylic acid sodium salt (VU0155041) offers a far more attractive alternative to investigate the neuroprotective potential of targeting mGlu4 receptors. Discovered by high-throughput screening, this lead compound was shown to be approximately 8-fold more potent than PHCCC at the mGlu4 receptor ( $EC_{50}$  of 0.7  $\mu$ M at rat mGlu4) assessed using thallium flux through G-protein regulated inwardly rectifying potassium channels (GIRK) (Niswender *et al.*, 2008a). In addition, VU0155041 did not demonstrate any significant potentiator or antagonist activity at other mGlu receptor subtypes (i.e. mGlu1, 2, 3, 5, 7 and 8), and is more readily soluble in aqueous vehicle (i.e. PBS) avoiding toxicity issues with DMSO (Niswender *et al.*, 2008). VU0155041 has also shown robust symptomatic activity in two rodent models of PD. Following intracerebroventricular (i.c.v.) administration of 31 and 316 nmol, VU0155041 dose dependently decreased haloperidol-induced catalepsy and reserpine-induced akinesia in rats (Niswender *et al.*, 2008). Therefore in the studies described in this chapter, we seek to investigate the contribution of mGlu4 in mediating neuroprotective effects, using the selective PAM VU0155041 in 6-OHDA lesioned rats.

In addition to showing enhanced glutamatergic signalling comparable to that in PD (Breit *et al.*, 2006; Hassani *et al.*, 1996), the 6-OHDA model also displays a pronounced inflammatory response including microglial activation in the striatum and SNpc and elevated striatal levels of inflammatory markers such as TNF- $\alpha$  (Cicchetti *et al.*, 2002; Duty & Jenner, 2011; Mogi *et al.*, 2000). Furthermore drugs with anti-inflammatory properties have been shown to provide neuroprotective effects in models of PD. Among these compounds, agonists of the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) such as pioglitazone, have been shown in pre-clinical studies to prevent dopaminergic cell loss induced by MPTP in mice (Breibert *et al.*, 2002; Dehmer *et al.*, 2004) and protect against inflammation-induced dopaminergic neurodegeneration by intrastriatal lipopolysaccharide (LPS) in rats (Hunter *et al.*,



2007). The antibiotic Minocycline has also been reported to attenuate MPTP-induced dopaminergic neurodegeneration by inhibiting MPTP-induced microglial activation, preventing the production of microglia-derived proinflammatory factors such as IL-1 $\beta$ , ROS and NO (Wu *et al.*, 2002). Taken together these findings suggest any neuroprotective effects mediated by mGlu4 receptors could involve an anti-inflammatory action. Furthermore, given that mGlu4 receptors have been identified on primary cultured rat astrocytes (Taylor *et al.*, 2003; Yao *et al.*, 2005), it is possible that these receptors may also reside on astrocytes in the rodent brain. These findings open up the possibility that glial-mediated anti-inflammatory actions in particular, may contribute to any protective effects mediated by mGlu4 receptor stimulation. Therefore in this chapter we also seek to establish the expression of mGlu4 receptors on both neuronal and astroglial cell types in rodent SNpc, and elucidate any potential effects of VU0155041 treatment on both astroglial and microglial activation in the 6-OHDA lesioned rat.

## 4.2 Aims

The overall aim of the studies described here was to examine whether selective activation of mGlu4 receptors using the selective mGlu4 PAM VU0155041 was sufficient to bring about functional neuroprotection in the 6-OHDA-lesion rat model of PD. In addition, studies were undertaken to begin to determine the potential mechanisms underlying this response, focussing especially on potential anti-inflammatory actions of VU0155041.

The primary aims of the studies outlined here were:

1. to determine if VU0155041 provided neuroprotective effects against a 6-OHDA lesion of the SNpc in rats, determined by immunohistochemical and neurochemical analysis
2. to ascertain whether neuroprotective effects of VU0155041 translated to functional preservation of motor function in both forced and habitual behavioural tasks
3. to determine if functional neuroprotection achieved with VU0155041 was lost on pre-treatment with a selective group III mGlu receptor antagonist CPPG
4. to delineate potential co-localisation of mGlu4 receptor subtypes on both neurones and astrocytes in naïve rat brain to begin to ascertain neuroprotective mechanisms
5. to investigate the effects of VU0155041 treatment on astrocytic and microglial activation post-lesion as a potential neuroprotective mechanism.

## **4.3 Methods**

### **4.3.1 Specific methodological details for VU0155041 neuroprotection studies**

#### **4.3.1.1 Animals**

Male Sprague Dawley rats (B & K or Harlan, U.K.) weighing 270 – 300 g were used in these studies. Food and water were provided *ad libitum*. Animals were housed in a temperature- and humidity-controlled environment with a 12-h light / dark cycle. All procedures conformed to the U.K. Animals (Scientific Procedures) Act, 1986 and every effort was made to minimise animal numbers and suffering.

#### **4.3.1.2 Surgical cannulation**

Animals were bilaterally cannulated in the same manner as previously described in section 3.3.2.4 whereby cannulae were implanted bilaterally 2mm above the SNpc using the following co-ordinates; anterioposterior (AP), -4.8mm, mediolateral (ML),  $\pm 2.0$ mm; dorsoventral (DV), -6.3mm from bregma (Paxinos & Watson, 1998).

#### **4.3.1.3 Supranigral drug administration**

A minimum of 5 days following cannulae implantation, the neuroprotection study commenced. Animals received their first dose of VU0155041 (10-100nmol, dissolved in 4 $\mu$ l PBS) or vehicle (4 $\mu$ l 1x PBS) 1 hour prior to 6-OHDA infusion (detailed in 4.3.1.4). Animals received unilateral drug infusions at a flow rate of 2 $\mu$ l min<sup>-1</sup> into the right cannulae (or left on occasions when the right was blocked) for a further 7 days post-lesion as previously described in section 3.3.2.3.

There are currently no mGlu4 selective antagonists available at this time therefore the broad spectrum antagonist CPPG was utilised to confirm neuroprotection mediated by VU0155041 was a receptor driven process. To investigate the effects of CPPG on neuroprotection mediated by VU0155041, CPPG (75 nmol; dissolved in 4 $\mu$ l 0.1M

NaOH) or its corresponding vehicle (4µl 0.1M NaOH) were infused at a rate of 2µl min<sup>-1</sup> unilaterally above the SNpc 30 min prior to each injection of VU0155041 (100nmol) using the same method described above.

#### **4.3.1.4 Intranigral 6-OHDA lesioning**

A minimum of 5 days following bilateral cannulation above the SNpc, animals received a single, unilateral injection of 6-OHDA into the same side of the SNpc as the drug infusions. 30 min prior to 6-OHDA injection, rats were pre-treated with desipramine (25 mg kg<sup>-1</sup> i.p.) and pargyline (5 mg kg<sup>-1</sup> i.p.). Animals were then anaesthetised briefly whilst 6-OHDA (12 µg in 2.5 µl of 0.2% ascorbic acid in 0.9% saline) was injected at a rate of 1.25 µl min<sup>-1</sup> into the SNpc (2 mm below the guide cannula) as previously described in section 3.3.2.4. Animals were placed in cages on thermostatically heated mats until fully conscious.

#### **4.3.1.5 Behavioural assessment**

The extent of motor impairment following a unilateral lesion to the SNpc was assessed at intervals using three behavioural tests. Animal behaviour was assessed using the adjusted steps test on day 3 and 6 post-lesion, cylinder test on day 5 post-lesioning, (both tests also required a baseline assessment one day prior to lesioning) and amphetamine induced rotations on day 7 post-lesion. Full details of these tests are given earlier in section 3.3.2.5.

#### **4.3.1.6 Data analysis for behavioural tests**

Only behavioural data from animals showing correct cannula placement were included for data analysis presented in these studies. All behavioural analyses were assessed in a similar manner to those described in section 3.3.2.6. Briefly, in the adjusted stepping test, use of the contralateral and ipsilateral paw in both forehand and backhand directions post lesion were expressed as a % of pre-lesion use. Adjusted stepping scores between treatment groups were then compared using a 2-way ANOVA with Dunnett's *post-hoc* test at both 3 and 6 days post-lesion. Use of

the contralateral and ipsilateral paw in the cylinder test, expressed as a % of total reaches, was also compared pre and post lesion between different treatment groups using a 2-way ANOVA with Bonferroni's *post-hoc* test. Finally, the number of amphetamine-induced rotations was plotted as both time course (5 min intervals) or total rotations between 15-45 min (when stable locomotor behaviour was achieved) and compared between treatment groups using a 2 or 1-way ANOVA respectively with a Bonferroni's *post-hoc* test. In all cases, data are expressed as mean  $\pm$  s.e.m. where  $P < 0.05$  was taken to be significant, with all statistical analysis performed using GraphPad Prism version 5.

#### **4.3.1.7 Immunohistochemical and HPLC protocols**

In the initial dose finding study in which behavioural tests were not undertaken, on the final day of VU0155041 dosing (day 8), a sub-set of animals were killed by CO<sub>2</sub> asphyxiation and brains were quickly removed for dissection of striatum and SN. Both SN and striatal tissue samples were then prepared for HPLC as previously described in section 3.3.2.9.

For the larger full study, on the final day of dosing (day 8), animals were killed by CO<sub>2</sub> asphyxiation, the brains removed and segmented into rostral (striatum) and caudal (SN) segments. The striata from these animals were immediately dissected from the rostral segment and snap frozen on card-ice before storing at -80°C until further processing for HPLC measurement of dopamine levels. The SN segment on the other hand, was placed in 4% formalin for a period of at least 2 days to ensure fixation before processing for immunohistochemistry. Once required, the nigral segment of the brain was dehydrated, defatted and then paraffin embedded. 6µm coronal sections were taken through the SN at -4.8, -5.3, and -5.8mm AP from bregma (Paxinos & Watson, 1986) as described in section 2.3.2.2.

TH and DDC immunohistochemistry in the SNpc was performed exactly as described in section 3.3.2.7. As before, staining results in the SN were viewed on a Zeiss apotome microscope and recorded using Axiovision LE software (Carl Zeiss Ltd., UK).

To investigate the effects of VU1055041 treatment on microglia and astrocytes, immunohistochemistry was performed on their respective markers ionised calcium binding adaptor molecule 1 (IBA-1) and glial fibrillary acidic protein (GFAP). Using 6µm sections from a central region of the SNpc in triplicate (-5.3mm AP from bregma, Paxinos & Watson, 1998) were deparaffinised and rehydrated as previously described in section 2.3.2.2. Sections were then immersed for 10min in 3% hydrogen peroxide to block endogenous peroxidase activity. Following this, slides were placed in pepsin (0.2 g Sigma-p-7000 pepsin in 50 ml 0.01 M HCl) for 30 min at RT, washed and nonspecific binding was blocked for 20 min with 1.5% normal goat serum (Vectastatin rabbit IgG ABC kit; Vector Laboratories, UK) diluted in PBS at RT. Sections were then incubated with primary rabbit polyclonal anti IBA-1 (1:5000; Wako Chemicals, USA) or anti- GFAP (pre-diluted neat solution; BioGenex Laboratories, USA) for 18 h at RT. After washing in PBS (3 X 5 min), sections were incubated with a biotinylated secondary antibody (Vectastatin rabbit IgG ABC kit) for 30 min at RT followed by 3 x 5 min washes with PBS. The horseradish peroxidase conjugate (Vectastatin rabbit IgG ABC kit) was then applied for 30 min followed by 3 x 5 min PBS rinses all at RT. Antibody binding was visualised by incubating sections with the chromagen 3, 3V-diaminobenzidine (DAB substrate kit, Vector SK-4100) for 10 min at RT. Sections were finally dehydrated in 100% IMS, cleared in xylene, cover-slipped with DPX mountant and allowed to dry before being analysed using light microscopy. Digital images of the SN were captured using an Aperio ScanScope XT scanner and analysed using Scion Image Software (Scion Corporation, USA).

Dopamine content of frozen striata was assessed using HPLC, exactly as described in Chapter 3, section 3.3.2.9.

#### **4.3.1.8 Image and data analysis for immunohistochemistry and HPLC studies**

For TH immunohistochemistry in the SNpc, the average number of TH-positive cells in three adjacent sections for each animal across the three rostrocaudal levels (-4.8, -5.3, -5.8mm AP from bregma) were obtained as previously described in section 3.3.2.8, and the mean of these values taken per treatment group. Statistical comparisons were made between VU0155041 and vehicle-treated animals of mean TH-positive cell counts in the 6-OHDA lesioned side, expressed as % of the contralateral, intact hemisphere, using a 1-way ANOVA with Dunnett's post-hoc test. For IBA-1 staining, the density of IBA-1-positive cells demonstrating amoeboid morphology typical of activated microglia, was assessed in the contralateral and ipsilateral hemispheres of the SNpc. GFAP positive cells demonstrating the hallmark star-shaped characteristic of an activated astrocyte were also compared between adjacent hemispheres at a central region of the SNpc (-5.3 mm AP from bregma). For each animal, three adjacent sections from the SNpc were counted and then pooled to give a total mean density/cell count for the ipsilateral and contralateral hemispheres. Statistical comparisons of mean IBA-1 density measures or GFAP-positive cell counts in the 6-OHDA lesioned hemisphere, expressed as a % of the contralateral, intact hemisphere were made between drug and vehicle-treated animals using a 1-way ANOVA with Dunnett's post-hoc test. For HPLC analyses, dopamine content of striatal and nigral samples were converted from peak areas of the chromatogram using a calibration curve of pure reference standards and expressed as ng.g<sup>-1</sup> protein. A 1-way ANOVA with Dunnett's *post-hoc* test was used to compare dopamine content of the 6-OHDA lesioned side, expressed as a % of the contralateral intact side between treatment groups. In all cases, data are expressed as mean  $\pm$  standard error of the mean (s.e.m.), where *n* represents the number of animals in each experimental group. Statistical analyses were performed using GraphPad Prism (version 5.0) and *P*<0.05 was taken to indicate significance.

#### **4.3.1.9 Immunofluorescence co-localisation protocol**

Immunohistochemical experiments using fluorescence were carried out to investigate co-localisation of mGlu4 with TH and GFAP. In each case mGlu4 receptors were

visualised using a three step indirect immunofluorescence method employing a biotinylated secondary antibody conjugated to a 594 fluorophore-streptavidin complex.

Two naïve rats were deeply anaesthetised using pentobarbital ( $100\text{mg kg}^{-1}$ , i.p.) and transcardially perfused with 200ml 100mM PBS followed by 200ml 4% paraformaldehyde (PFA) in PBS. Brains were quickly removed and placed in fixative overnight to ensure complete fixation. The brain was then cut into a rostral (striatal) and caudal (SN) segment, dehydrated and defatted before being manually embedded into paraffin wax. Coronal sections ( $6\mu\text{m}$ ) at a central region of the SNpc (-5.3mm AP from bregma), according to the rat brain atlas (Paxinos & Watson., 1998) were cut on a microtome and taken up onto slides as previously described in section 2.3.2.2.

For each immunofluorescence co-localisation study, three adjacent sections containing the SN (-5.3mm AP) were de-paraffinised in xylene and 100% industrial methylated spirit (IMS) before a 10-min incubation in 3% hydrogen peroxide to block endogenous peroxide activity. Following standard antigen retrieval with 1mM citric acid then a 10-min incubation with blocking buffer (1% BSA in 0.1M PBS and 10% sodium azide), sections were incubated with the primary antibody rabbit polyclonal anti-mGlu4 primary antibody (Abcam, 1:50) at RT overnight to localise mGlu4 receptors. Sections were then washed in TBS, and then incubated for 1 hour with a goat anti-rat biotinylated secondary antibody (Sigma). Thereafter, sections were washed with TBS and incubated for 1 hour at room temperature with a fluorescent AlexaFluor 594 streptavidin complex (Invitrogen, USA).

To assess co-localisation with TH or GFAP, sections were then washed with TBS and subsequently incubated with a mouse monoclonal anti-TH (MAB318, Chemicon, 1:1000), or anti-GFAP (Sigma, G3893, 1:1000), primary antibody for 1 hour at RT. Thereafter, sections were washed with TBS, and then incubated for 1 hour at RT with a goat anti-mouse-488 fluorescent antibody (Alexa Fluor, Invitrogen, 1:1000) solution containing Hoechst (Sigma, 33258, 1: 5000). The immunofluorochemically stained rat brain sections were then washed in TBS, and immediately mounted with



glass cover slips using the hydrophilic anti-fade mountant, mowiol 4-88. Fluorescent images of sections were captured using a Zeiss Apotome fluorescent microscope and analysed for co-localisation using Mosaic and Z-stack multicolour functions using Axiovision image analysis software.

#### **4.3.2.10 Materials and suppliers**

Details of all reagents, consumables and supplier contacts are included in Appendix I.

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## 4.4 Results

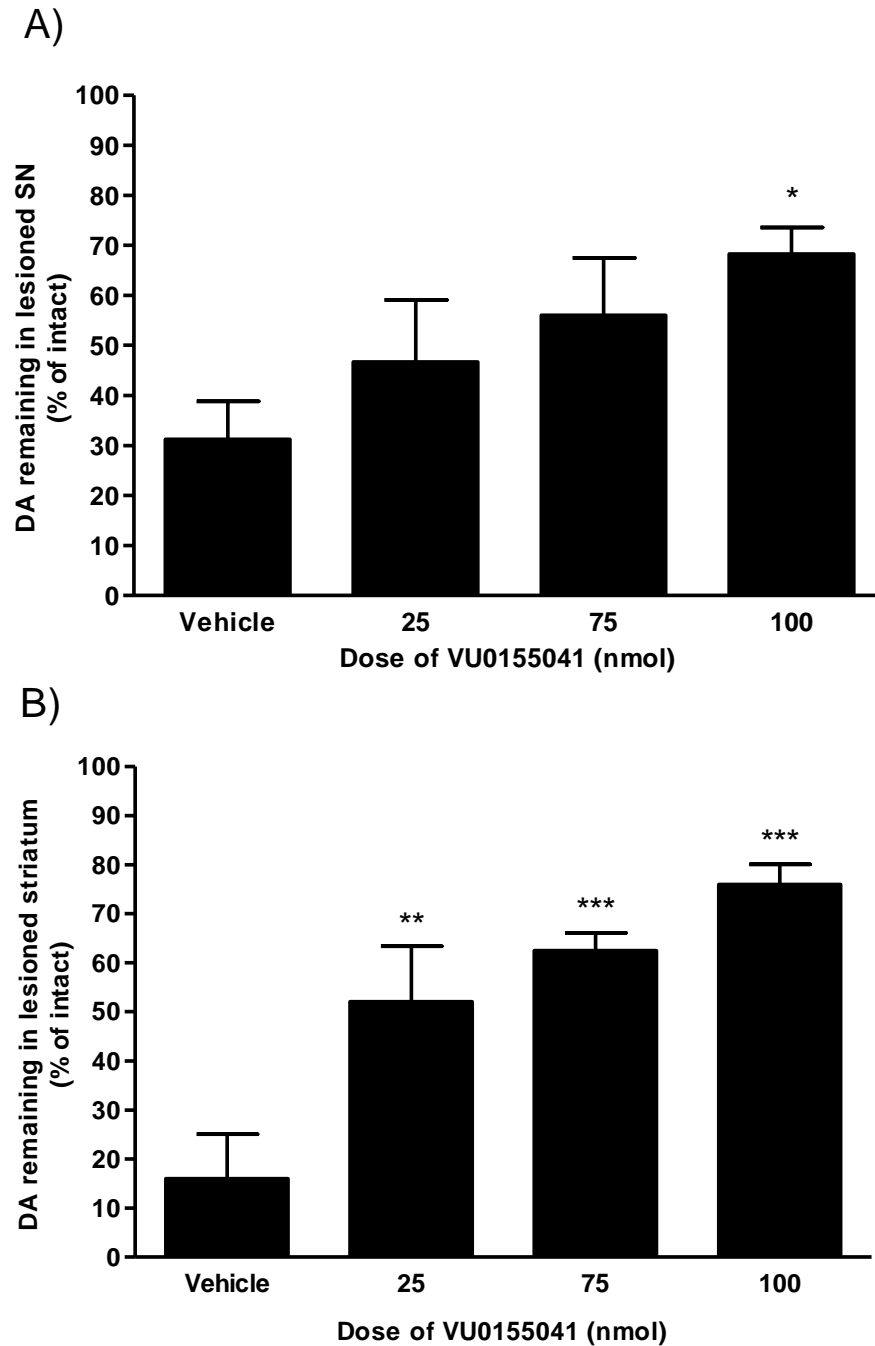
### 4.4.1 Initial dose finding study to investigate the effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, in unilaterally 6-OHDA-lesioned rats

#### 4.4.1.1 Effects of sub-chronic VU0155041 infusion on dopamine content in the substantia nigra and striatum

In an initial dose finding study using HPLC, nigral dopamine content was shown to be preserved in the lesioned SN of animals treated with VU0155041 (figure 4.1). In vehicle-treated 6-OHDA lesioned animals, nigral dopamine content fell from  $2430 \pm 109 \text{ ng g}^{-1}$  in the intact hemisphere to  $750 \pm 180 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $31.2 \pm 7.6\%$  ( $n=6$ ) of the intact hemisphere (figure 4.1A). VU0155041 treatment produced a dose-dependent preservation of nigral dopamine content, reaching a maximal significant effect with 100nmol VU0155041, where dopamine content in the lesioned hemisphere equated to  $68.3 \pm 5.3\%$  of the intact hemisphere ( $P < 0.05$  versus vehicle treatment; 1-way ANOVA with Dunnett's *post-hoc* test).

A more profound neuroprotective effect was observed on assessment of striatal dopamine following VU0155041 treatment. In vehicle-treated 6-OHDA lesioned animals, striatal dopamine content fell from  $17144 \pm 758 \text{ ng g}^{-1}$  in the intact hemisphere to  $2680 \pm 1578 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $16.0 \pm 9.1\%$  ( $n=6$ ) of the intact hemisphere (figure 4.1B). VU0155041 treatment at the lowest dose tested (25nmol) significantly preserved levels of striatal dopamine content equating to  $52.1 \pm 11.3\%$  ( $n=6$ ) of the intact hemisphere. A subsequent dose-dependent increase in preservation of striatal dopamine content was observed reaching maximal effect with 100nmol VU0155041, where dopamine content in the lesioned hemisphere remained at  $76.0 \pm 4.2\%$  of the intact hemisphere ( $P < 0.001$  versus vehicle treatment; 1-way ANOVA with Dunnett's *post-hoc* test).

Figure 4.1: Effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, on dopamine content in the substantia nigra and striatum of unilaterally 6-OHDA lesioned rats.



**Figure 4.1.** The effects of sub-chronic supranigral infusion of VU0155041 (25-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the on the 6-OHDA induced loss of dopamine content in the SN (**A**) and striatum (**B**) on the lesioned side expressed as a percentage of the intact side. For both, values represent mean  $\pm$  s.e.m. (n=6 for all treatment groups). \* (P<0.05), \*\* (P<0.01) and \*\*\* (P<0.001) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). For (**A**) F = 2.640, df = 3,20 and for (**B**) F = 10.99, df = 3,20.

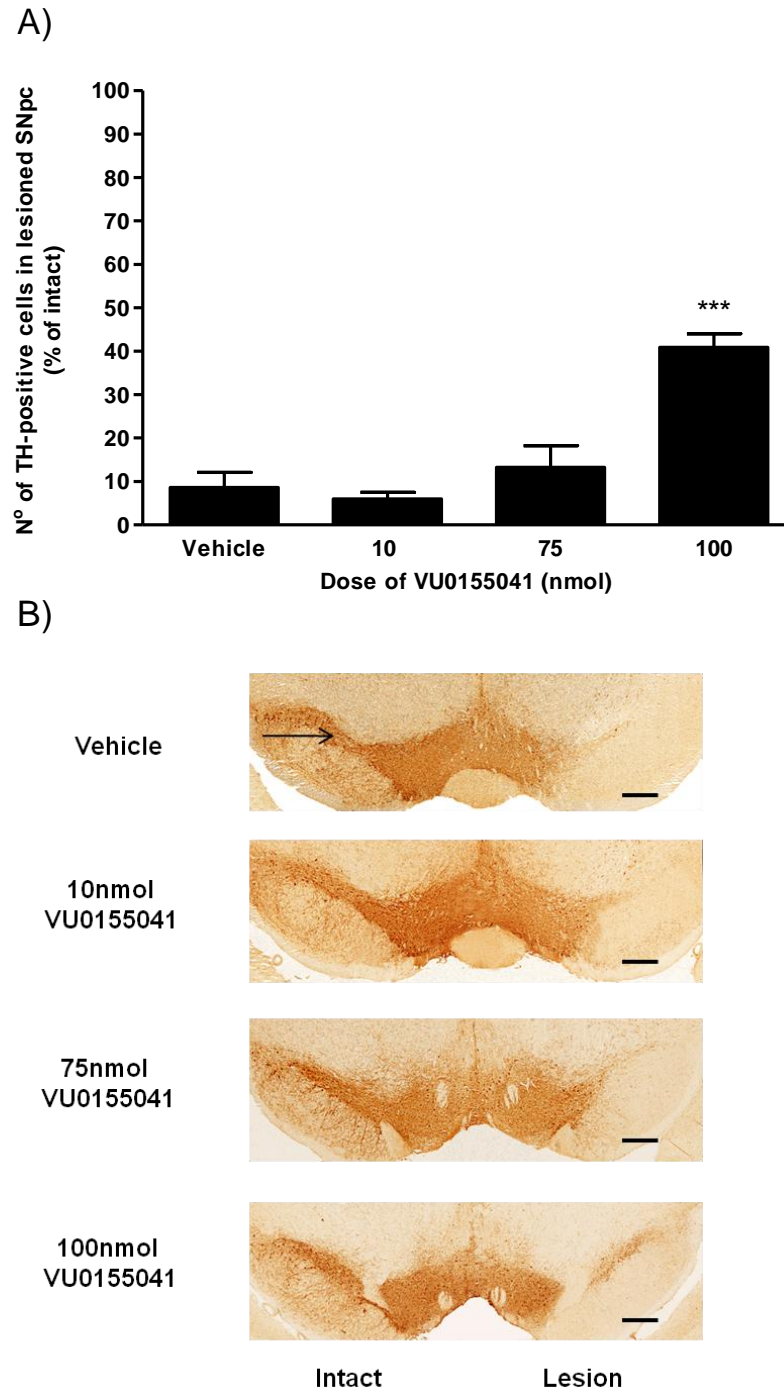
#### **4.4.2 Full neuroprotection study to investigate effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, in unilaterally 6-OHDA-lesioned rats**

##### **4.4.2.1 Effects of sub-chronic VU0155041 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta**

In a separate follow-up full neuroprotection study, the effects of VU0155041 were assessed using TH immunohistochemistry. In the SNpc, the mean sum of TH-positive cells in triplicate sections obtained across three rostrocaudal levels declined from  $306.3 \pm 7.4$  in the intact hemisphere to  $26.5 \pm 5.7$  in the lesioned hemisphere of vehicle-treated 6-OHDA lesioned animals ( $n=8$ ), demonstrating a full lesion with a deficit  $> 90\%$ . Owing to the considerable preservation of dopamine content observed following 25nmol VU0155041 in the initial study, a lower (10nmol) dose of VU0155041 was investigated here to achieve a fuller neuroprotective dose response profile. Supranigral treatment with VU0155041 significantly protected against TH-positive cell loss whereby maximal protection was reached with a 100nmol dose of VU0155041. The number of TH-positive cells remaining in the lesioned hemisphere following 100nmol VU0155041 treatment ( $n=6$ ), was  $131.3 \pm 11.6$  equivalent to  $40.9 \pm 3.1\%$  of the intact hemisphere in contrast to  $8.6 \pm 3.5\%$  remaining in the vehicle-treated animals ( $n=8$ ,  $P < 0.001$ ; one-way ANOVA with a Dunnett's *post-hoc* test; figure 4.2A).

Representative nigral sections demonstrating immunoreactivity to TH-positive cells in the 6-OHDA lesioned animals treated with sub-chronic VU0155041 (10-100nmol) are shown in figure 4.2B. The photomicrographs demonstrate supranigral infusion of VU0155041 (100nmol) clearly provides noticeable preservation of TH-positive cells in the lesioned hemisphere. Little effect on TH-positive cells was observed with a low dose of VU0155041 (10nmol), however a degree of preservation was observed on increasing the dose of VU0155041 (75nmol) compared to vehicle treated animals, consistent with a graded dose response profile.

Figure 4.2 Effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator VU0155041, on tyrosine hydroxylase-positive cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.



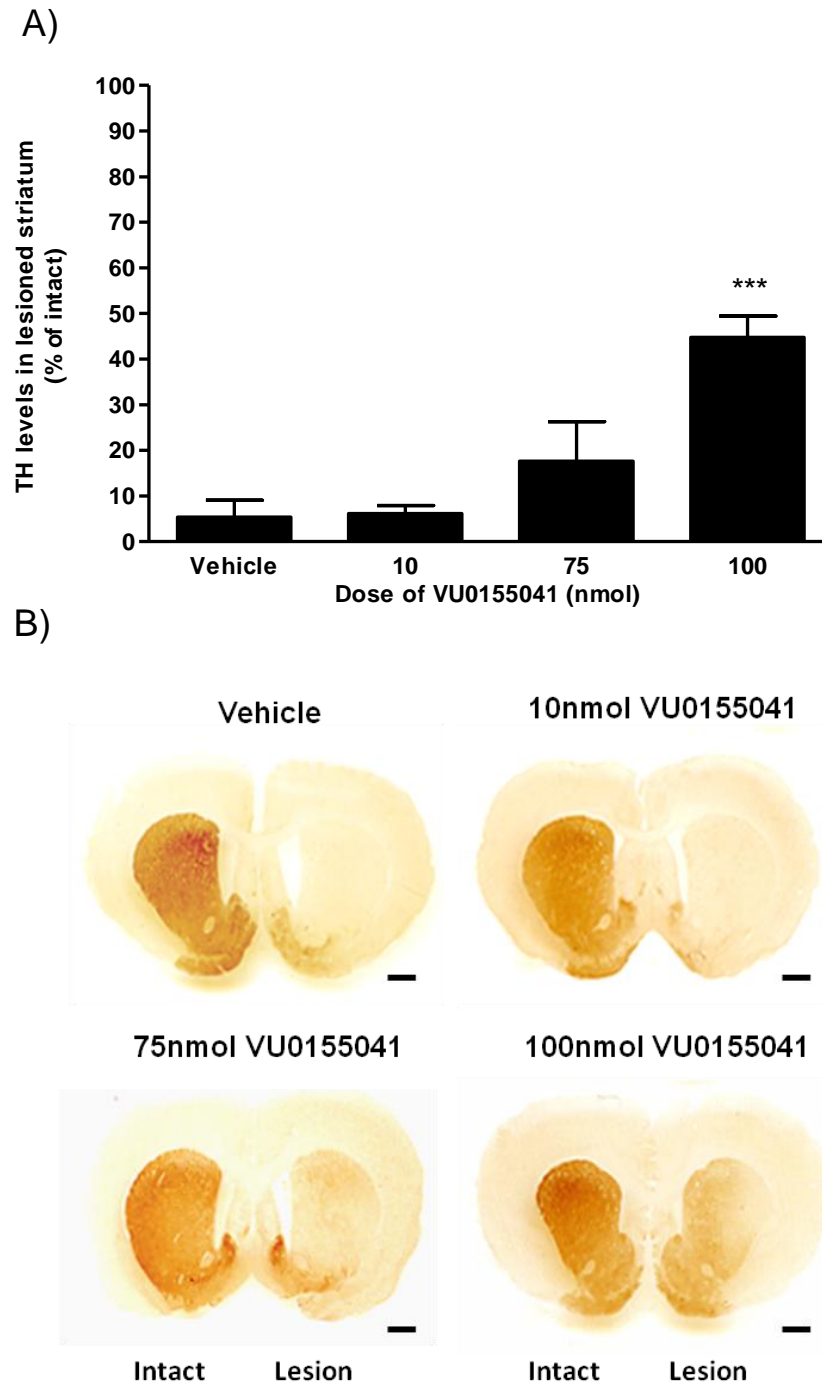
**Figure 4.2.** (A) The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of TH-positive cells in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \*\*\* (P < 0.001) indicate significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 16.66; df = 3,25. (B) Representative photomicrographs showing the levels of TH immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic VU0155041 treatment. Arrow delineates SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **4.4.2.2 Effects of sub-chronic VU0155041 infusion on tyrosine hydroxylase levels in the striatum of 6-OHDA lesioned rats**

The optical density measurements of TH immunoreactivity in striatal sections from animals treated supranigrally with sub-chronic VU0155041 (10-100nmol) and those treated with vehicle is quantified in figure 4.3. When the combined mean striatal TH optical density was quantified, TH levels declined in the lesioned striatum of subsequent vehicle-treated animals to  $5.3 \pm 3.7\%$  of the intact side. Supranigral infusion of VU0155041 (10-100nmol), 1 hour prior and for 7 days after 6-OHDA infusion dose dependently preserved striatal TH immunoreactivity in the lesioned hemisphere obtaining maximal effect with the highest dose of VU0155041 (100nmol) tested. Following 100nmol VU0155041, significant preservation of TH immunoreactivity was observed where the overall mean optical density in the lesioned striatum amounted to  $44.8 \pm 4.7\%$  of the intact hemisphere, suggesting dopaminergic striatal nerve terminals were considerably protected in these animals ( $n=6-8$ ;  $P<0.001$  versus vehicle treatment; 1-way ANOVA with Dunnett's *post-hoc* test; figure 4.3A).

Representative striatal sections showing TH immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic VU0155041 (10-100nmol) are shown in figure 4.3B. The photomicrographs demonstrate VU0155041 at the highest dose tested (100nmol), provides extensive preservation of striatal immunoreactivity in comparison to vehicle-treated animals.

Figure 4.3: Effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, on tyrosine hydroxylase immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



**Figure 4.3.** (A) The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of TH immunoreactivity in the striatum expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10 and n= 6 for 100nmol VU0155041) \*\*\* (P <0.001) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post- hoc* test). F = 9.475; df = 3,25. (B) Representative photomicrographs showing the levels of TH immunoreactivity in striatal sections from 6-OHDA-lesioned animals following sub-chronic VU0155041 treatment. Lesion side on the right; scale bar: 500 $\mu$ m.

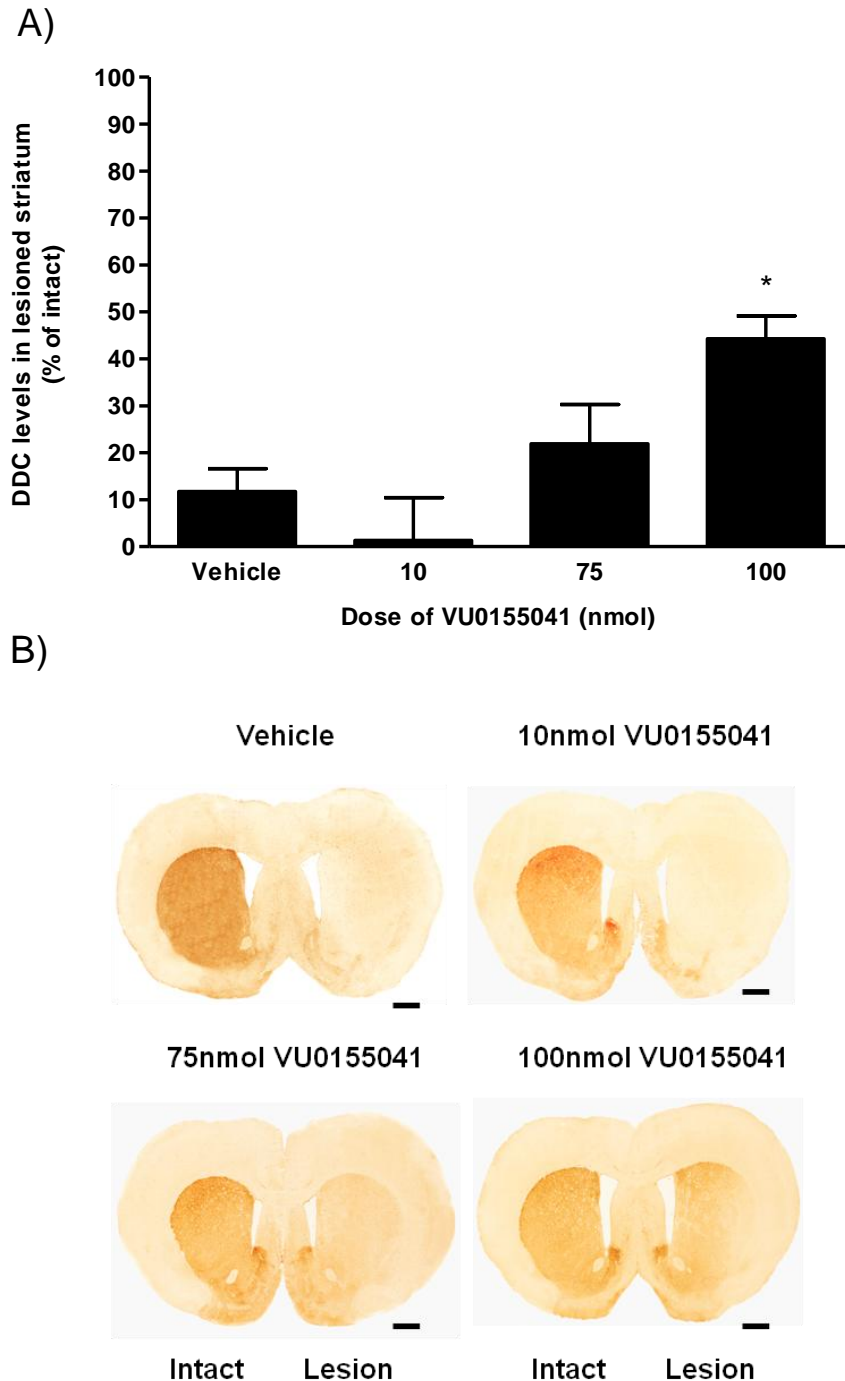
#### **4.4.2.3 Effects of sub-chronic VU0155041 infusion on dopa decarboxylase in the striatum of 6-OHDA lesioned rats**

The optical density of DDC immunoreactivity in striatal sections from animals treated supranigrally with sub-chronic VU0155041 (10-100nmol) and those treated with vehicle are shown in figure 4.4. When the combined mean striatal DDC optical density was quantified, DDC levels declined in the lesioned striatum of subsequent vehicle-treated animals to  $11.7 \pm 4.9\%$  of the intact side. Supranigral infusion of VU0155041, 1 hour prior and for 7 days after 6-OHDA infusion, dose dependently preserved striatal immunoreactivity in the lesioned hemisphere. Maximum protection was observed following 100nmol VU0155041 where the overall mean optical density in the lesioned striatum amounted to  $44.3 \pm 4.9\%$  of the intact hemisphere, demonstrating striatal DDC immunoreactivity was also significantly preserved with respect to vehicle-treated animals ( $n=6-8$ ;  $P<0.05$ ; 1-way ANOVA with Dunnett's post-hoc test; figure 4.4A). This finding confirmed VU0155041 treatment preserved dopaminergic striatal nerve terminals in these animals and that preserved levels of TH was not a mere reflection of increased expression of the marker.

Representative striatal sections showing DDC immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic VU0155041 (10-100nmol) are shown in figure 4.4B. The photomicrographs demonstrate supranigral infusion of VU0155041 at the highest dose tested (100nmol), provided noticeable protection of striatal immunoreactivity when compared to vehicle treated animals.



Figure 4.4: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric modulator, VU0155041, on dopa decarboxylase immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



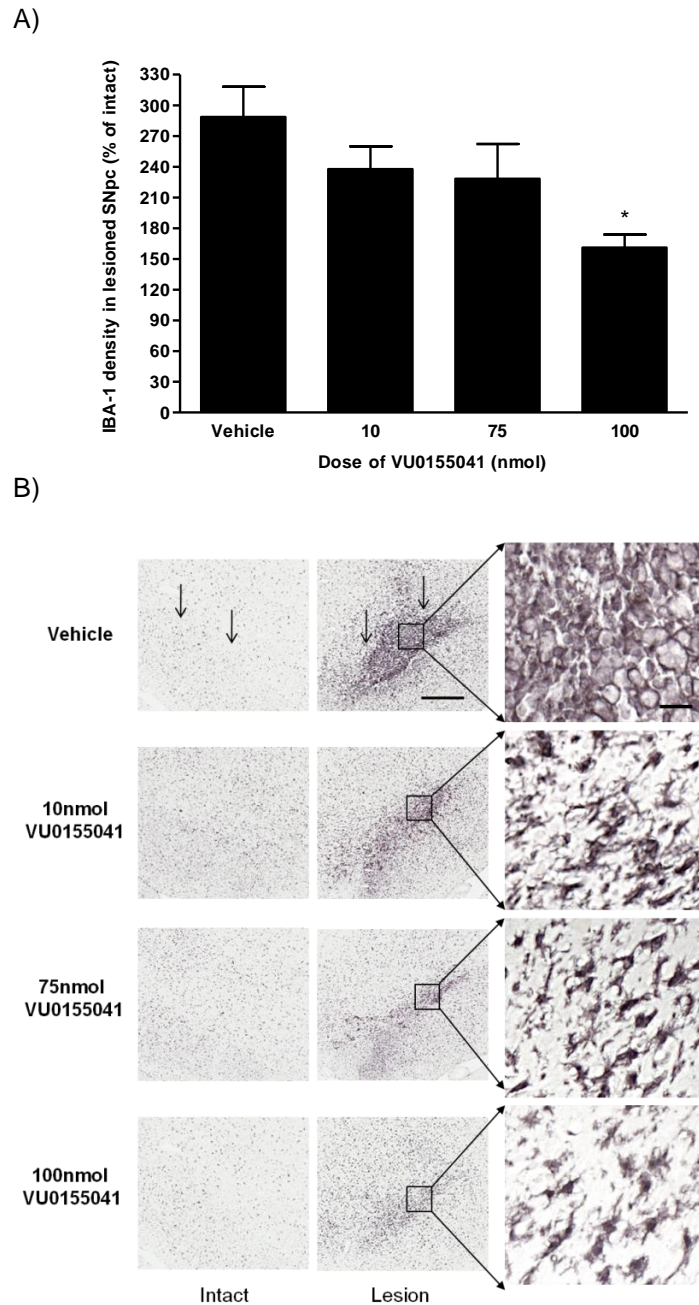
**Figure 4.4.** (A) The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of DDC in the striatum on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \* (P < 0.05) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 5.738; df = 3,25. (B) Representative photomicrographs showing the levels of DDC immunoreactivity in striatal sections from 6-OHDA-lesioned animals following sub-chronic VU0155041 treatment. Lesion side on the right; scale bar: 500 $\mu$ m.

#### **4.4.2.4 Effects of sub-chronic VU0155041 infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta**

Analysis of IBA-1, revealed a unilateral injection of 6-OHDA markedly increased microglial activation in the lesioned SNpc compared to the intact non-lesioned hemisphere (figure 4.5A). Following a unilateral 6-OHDA lesion, the mean total IBA-1 density at a central region of the SNpc increased to ~ 289% of the intact hemisphere in vehicle treated animals (n=8). However, following VU0155041 treatment (100nmol, n=6) this increase in IBA-1 density was significantly reduced compared to vehicle treated animals, whereby only a 161% increase in IBA-1 immunoreactivity was observed with respect to the intact hemisphere ( $p<0.05$ ; one-way ANOVA with Dunnett's *post-hoc* test).

Representative nigral sections showing IBA-1 immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic VU0155041 (10-100nmol) are shown in figure 4.5B. Qualitative analysis revealed IBA-1-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in IBA-1-positive cells was observed, with a switch from ramified to amoeboid morphology. The photomicrographs demonstrate supranigral infusion of VU0155041 dose-dependently reduced levels of IBA-1 compared to vehicle treated animals in tune with a decreased inflammatory response.

Figure 4.5: Effects of sub-chronic supranigral infusion of the selective mGlu4 positive allosteric modulator, VU0155041, on ionised calcium binding adaptor molecule 1 immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



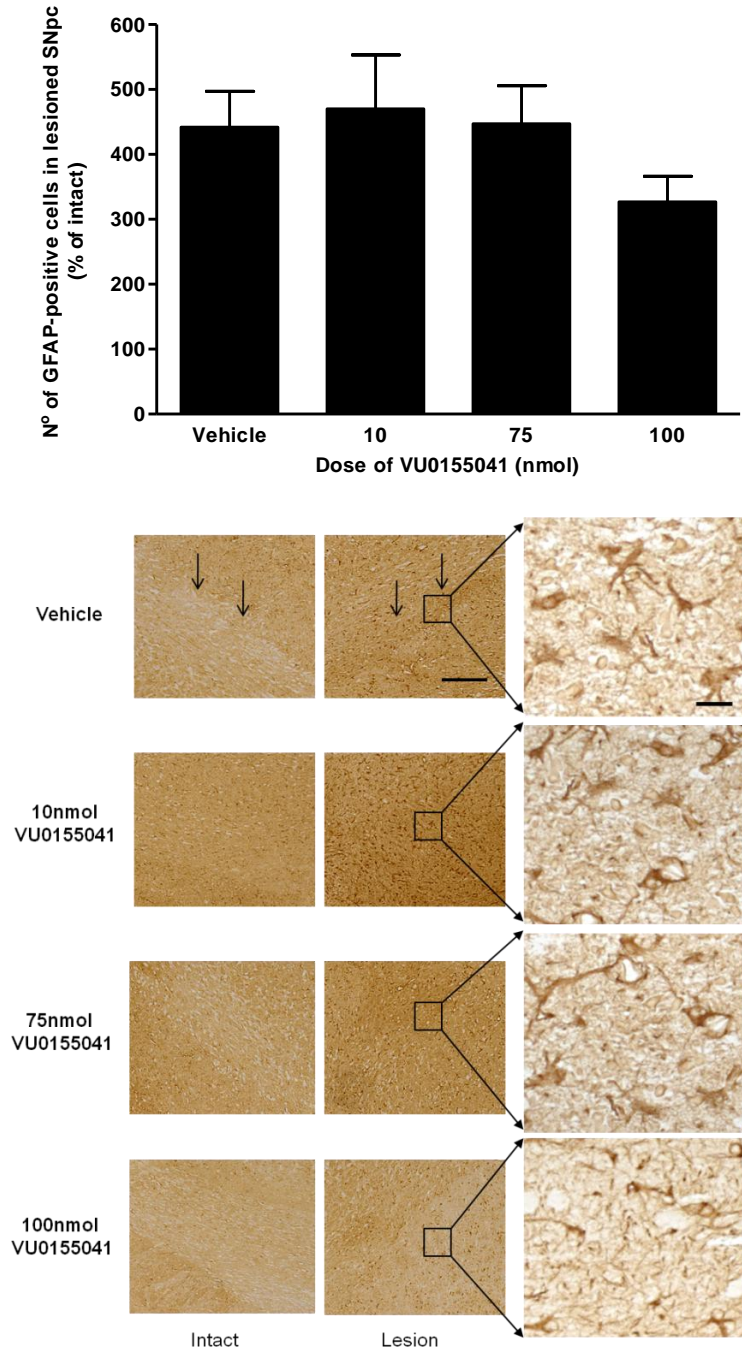
**Figure 4.5. (A)** The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced elevation of IBA-1 density in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \* ( $P < 0.05$ ) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test).  $F = 3.345$ ;  $df = 3,25$ . **(B)** Representative photomicrographs demonstrating IBA-1 immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic VU0155041 treatment. Lesion side on the right; scale bar: 200 $\mu$ m on main panel, 50 $\mu$ m on higher magnification images. Arrows indicate the dorsal border of the SNpc.

#### **4.4.2.5 Effects of sub-chronic VU0155041 infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta**

A marked increase in the astrocytic marker and hallmark of neurotoxicity, GFAP, was also observed in the lesioned SNpc compared to the intact non-lesioned hemisphere in vehicle-treated animals (figure 4.6A). Following a unilateral 6-OHDA lesion, the mean total number of GFAP- positive cells increased to 442% of the intact hemisphere in vehicle treated animals (n=8). Following VU0155041 treatment (100nmol, n=6), the increase in GFAP immunoreactivity in the lesioned SNpc was markedly reduced on comparison to vehicle treated animals, where a 327% increase in GFAP-positive cells was observed with respect to the intact hemisphere. This effect however was not significant ( $p > 0.05$ ; one-way ANOVA with Dunnett's *post-hoc* test).

Representative nigral sections demonstrating immunoreactivity to GFAP-positive cells in the 6-OHDA lesioned animals treated with sub-chronic VU0155041 (10-100nmol) are shown in figure 4.6B. Qualitative analysis revealed GFAP-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in GFAP-positive cells displaying thickened star-shaped morphology was observed. The photomicrographs confirm what was noted above, that supranigral infusion of VU0155041 (100nmol) provided a marked reduction in GFAP activation compared to vehicle treated animals, despite this effect not reaching significance.

Figure 4.6: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric modulator VU0155041, on glial fibrillary acidic protein cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.



**Figure 4.6. (A)** The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on GFAP-positive cell number in the SNpc following a 6-OHDA lesion expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \* (P < 0.05) indicates significant differences compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 0.9321; df = 3,25. **(B)** Representative photomicrographs demonstrating GFAP immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic VU0155041 treatment. Lesion side on the right; scale bars: 200 $\mu$ m on main panel, 50 $\mu$ m on higher magnification images. Arrows indicate the dorsal border of the SNpc.

#### **4.4.2.6 Effects of sub-chronic VU0155041 infusion on motor behaviour in 6-OHDA lesioned rats**

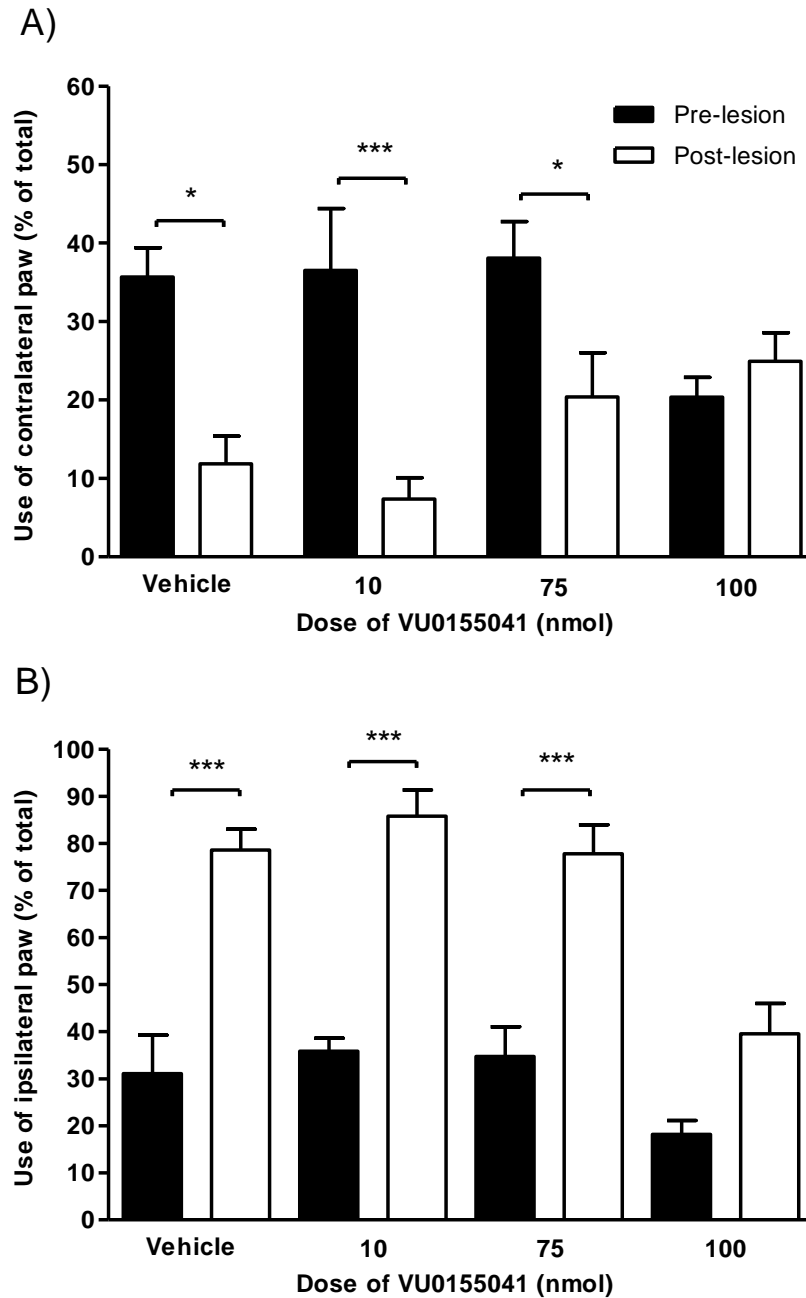
##### *Cylinder Test*

The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats were assessed using the cylinder test on day 5 post lesion.

As expected in vehicle treated animals, there was a significant decrease in use of the contralateral paw from  $35.7 \pm 3.7\%$  pre-lesion to  $11.9 \pm 3.5\%$  post-lesion confirming that a unilateral 6-OHDA lesion induced a reduction of approximately 68% in contralateral paw use (figure 4.7A). VU0155041 (10-75nmol) failed to significantly protect contralateral paw use although improvements in post-lesion scores were observed following 75nmol VU0155041. Pre-lesion scores in animals subsequently treated with 100nmol VU0155041 (~20%) were below that normally expected (~30%). However, in these animals subsequent assessment of post-lesion scores alone revealed a marked improvement in contralateral paw use following this high dose of VU0155041 with respect to vehicle-treated animals. Furthermore in VU0155041 (100nmol) treated animals, use of the contralateral paw was almost equivalent to that observed prior to a 6-OHDA lesion demonstrating total preservation. Contralateral paw use equated to  $25.0 \pm 2.5\%$  of total paw use post-lesion in comparison to  $20.4 \pm 2.5\%$  pre-lesion, demonstrating approximately an 18% increase in contralateral paw use post-lesion following treatment with 100nmol VU0155041.

A marked increase in ipsilateral paw use was also observed in vehicle treated animals following a 6-OHDA lesion in tune with the significant decrease in contralateral paw use. However with increasing doses of VU0155041 and subsequent increases in use of contralateral paw (as described above) use of the ipsilateral paw decreased. For example in vehicle treated animals, ipsilateral paw use post lesion increased by >150% with respect to pre-lesion score, compensating for an impaired contralateral paw. However in VU0155041 treated animals (100nmol), ipsilateral paw use post lesion increased by < 120% of pre-lesion score confirming the reduced asymmetry in these animals (figure 4.7B).

Figure 4.7: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric potentiator VU0155041, on motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



**Figure 4.7.** The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on contralateral (A) and ipsilateral (B) paw use expressed as a percentage of total paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion. For all, values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041) \* (P < 0.05) and \*\*\* (P < 0.001) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) pre/post lesion factor, F = 18.73, df = 1,50 and for treatment factor, F = 1.120, df = 3,50. In (B) pre/post lesion factor, F = 94.87, df = 1,50 and for treatment factor, F = 10.64, df = 3, 50.

### *Adjusted Stepping*

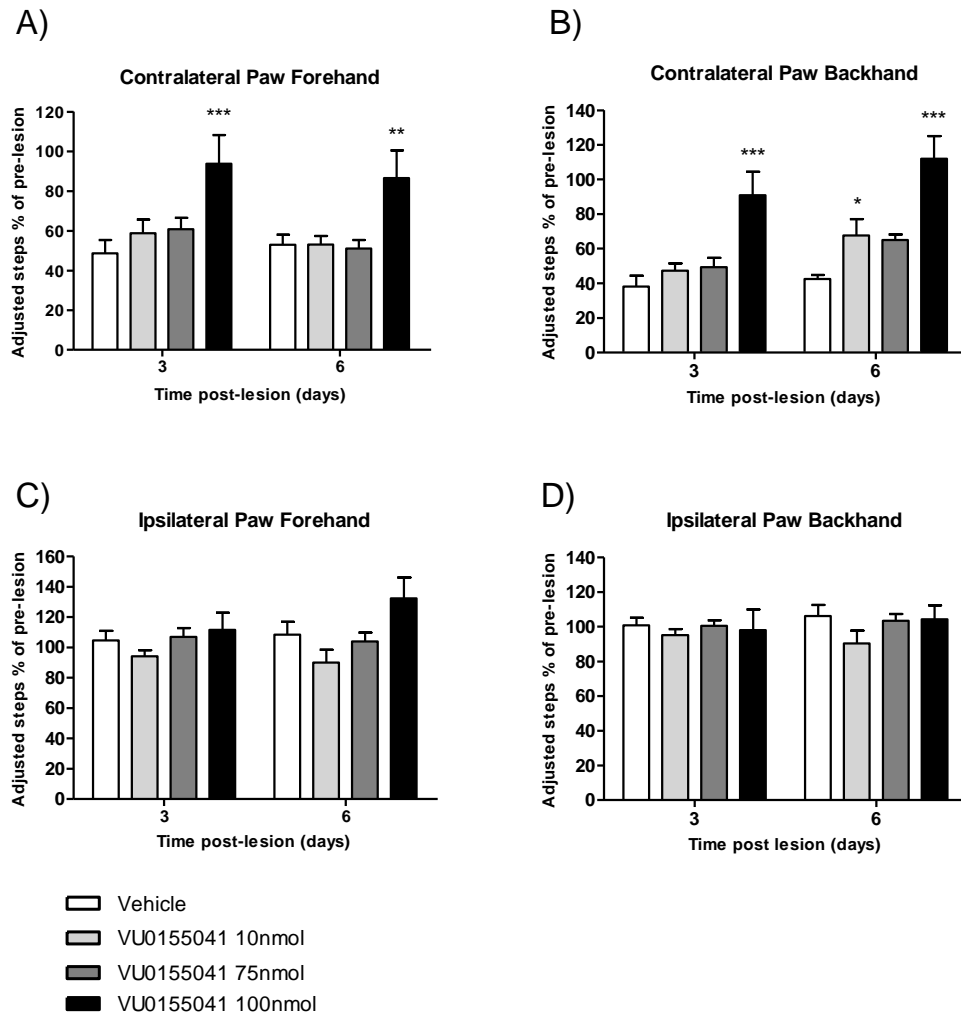
The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 4.8. As expected, the contralateral paw (lesioned) demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $48.7 \pm 6.7\%$  at 3 days post-lesion and  $52.9 \pm 5.2\%$  at 6 days in the vehicle treated group, indicating unilateral 6-OHDA lesioning reduced the number of adjusted steps in this direction (figure 4.8A). Infusion of the two lower doses of VU0155041 failed to significantly modify this loss of contralateral paw stepping. However, VU0155041 (100nmol) almost totally preserved the decline in contralateral paw use where scores equated to  $93.8 \pm 14.5\%$  of pre-lesion scores at 3 days post-lesion and  $86.5 \pm 13.9\%$  at 6 days, both of which were a significant effect with respect to vehicle-treated animals ( $P < 0.001$  and  $P < 0.01$  respectively).

In the backhand direction, there was also a marked reduction in contralateral paw use in vehicle treated animals. This amounted to a decrease in adjusted steps from 100% pre-lesion to  $38.2 \pm 6.3\%$  at 3 days post-lesion and  $42.6 \pm 2.3\%$  at 6 days confirming unilateral 6-OHDA-lesioning also reduced the number of adjusted steps in the backhand direction (figure 4.8B). However following VU0155041 treatment use of the contralateral paw in the backhand direction was also significantly preserved, attaining maximal effect with the 100nmol dose, whereby  $90.9 \pm 13.5\%$  of pre-lesion scores at 3 days and  $112.1 \pm 13.1\%$  at 6 days were obtained. Collectively these findings demonstrate supranigral sub-chronic VU0155041 (100nmol) treatment reduced the motor asymmetry induced by a unilateral 6-OHDA lesion shown here by preserving contralateral adjusted stepping in both forehand and backhand directions. In the case of the backhand direction, the effect of VU0155041 treatment was of such magnitude to restore contralateral paw use to pre-lesion levels.

In the ipsilateral (non-lesioned) paw in both the forehand and backhand direction no significant changes in adjusted steps 3 or 6 days post-lesion with respect to vehicle treated animals was observed (figure 4.8C and D).



Figure 4.8: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric potentiator VU0155041, on motor function assessed using the adjusted stepping test in unilaterally 6-OHDA lesioned rats.



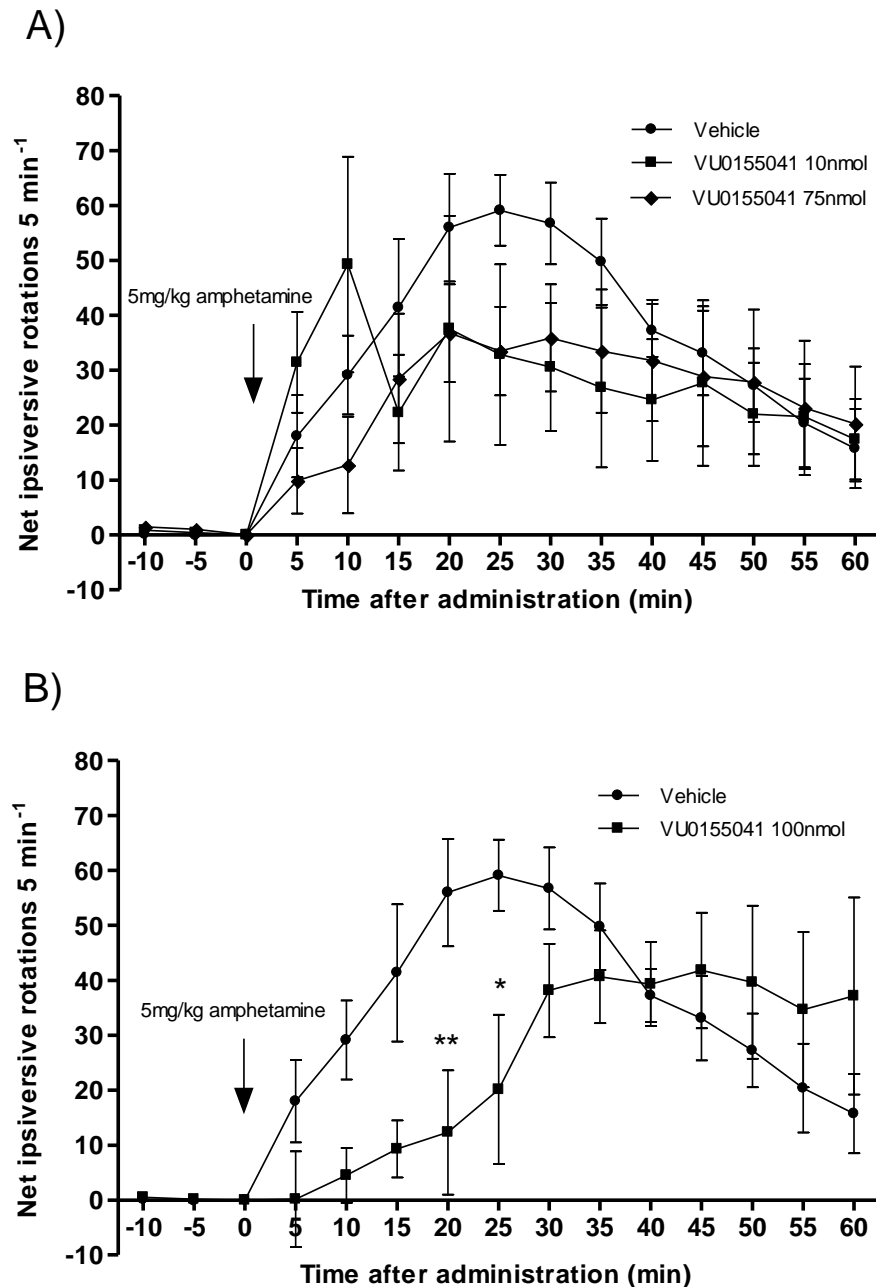
**Figure 4.8.** The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4μl) or vehicle (4μl PBS) on contralateral and ipsilateral paw use in the forehand and backhand direction (A-D) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion expressed as a percentage of pre-lesion score. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \*(P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for treatment factor, F = 9.676, df = 3,50 and for days post lesion factor, F = 0.7086, df = 1,50. In (B) treatment factor, F = 22.62, df = 3,50 and for days post lesion factor, F = 8.591, df = 1,50. In (C) treatment factor, F = 4.112, df = 3, 50 and for days post lesion factor, F = 0.5964, df = 1,50. In (D) treatment factor, F = 1.245, df = 3, 50 and for days post lesion factor, F = 0.3067, df = 1,50.

### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic VU0155041 or vehicle are shown in figure 4.9. Both graphs A and B show the same vehicle group alongside varying doses of VU0155041 for clarity, but all groups were analysed together. Analysis of time-course in vehicle treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. However, in animals treated with VU0155041 a marked reduction in amphetamine-induced ipsiversive rotations was noted. Figure 4.9A demonstrated 10 and 75nmol doses of VU0155041 induced a moderate reduction in ipsiversive rotations between 15 and 35 min post injection of amphetamine, although neither effect was significant compared to vehicle-treated animals ( $P > 0.05$ ; 2-way ANOVA with Bonferroni's *post-hoc* test). VU0155041 (100nmol) treated animals demonstrated the greatest reduction in ipsiversive rotations reaching a significant difference at 20 and 25 min post injection of amphetamine ( $P < 0.01-0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test) compared to vehicle-treated animals (figure 4.9B).

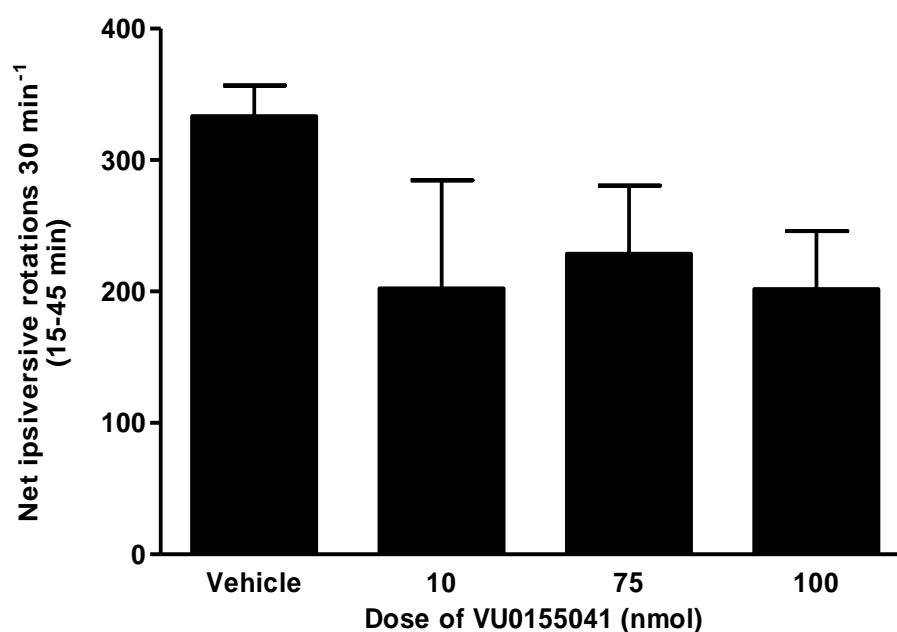
In figure 4.10, the mean total ipsiversive rotations were quantified over a 30 min period (15-45 min). The number of ipsiversive rotations by amphetamine ( $5\text{mg.kg}^{-1}$ ) in vehicle treated animals was  $333 \pm 24$  rotations in  $30\text{ min}^{-1}$ . VU0155041 markedly decreased amphetamine-induced ipsiversive rotations where at 100nmol VU0155041, the total mean number of rotations equated to  $202 \pm 44$ , demonstrating a 39% reduction compared to vehicle-treated animals, however this result was not significant ( $P > 0.05$ , 1-way ANOVA with Dunnett's *post-hoc* test).

Figure 4.9: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric modulator VU0155041, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 4.9.** The effects of sub-chronic supranigral infusion of VU0155041 (10-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. **(A)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and VU0155041 (10, 75nmol) treated animals. **(B)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and VU0155041 (100 nmol) treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041). \* (P <0.05), \*\* (P <0.01) indicates significant differences between VU0155041 (100nmol) and vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). For treatment factor F = 2.546; df = 3, 375 and for time F = 8.129; df = 14, 375.

Figure 4.10: Effects of sub-chronic supranigral infusion of the selective mGlu4 receptor positive allosteric modulator VU0155041, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 4.10.** Total ipsiversive rotations induced by amphetamine in 30 min<sup>-1</sup> (15-45 min) in vehicle and VU0155041 treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle and 75nmol VU0155041, n=7 for 10nmol VU0155041, n=6 for 100nmol VU0155041).  $F = 1.406$ ;  $df = 3, 25$ .

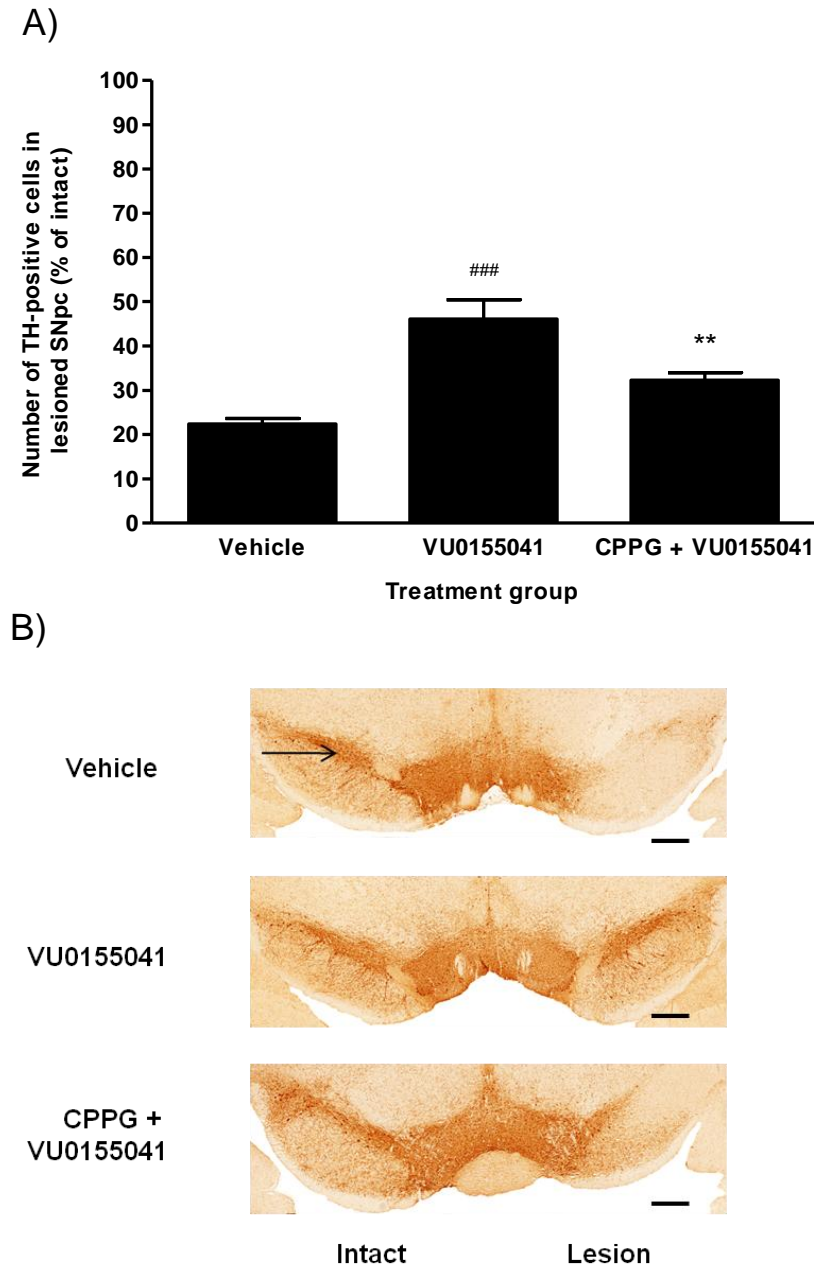
### **4.4.3 Effects of pre-treatment with group III mGlu receptor antagonist CPPG, on VU0155041 mediated neuroprotection in unilaterally 6-OHDA-lesioned rats**

#### **4.4.3.1 Effects of pre-treatment with CPPG on VU0155041 mediated preservation of tyrosine hydroxylase positive cells in the substantia nigra pars compacta**

The protective effects of VU0155041 (100nmol) in preserving TH-positive cells in the SNpc following a unilateral 6-OHDA lesion were found to be significantly inhibited by pre-treatment with CPPG (75nmol). In this study, 100nmol VU0155041 mediated significant preservation of TH-positive cells in the lesioned SNpc amounting to  $46.1 \pm 4.4\%$  of the intact hemisphere effectively reproducing the level of preservation achieved in the previous study ( $44.8 \pm 4.7\%$  of the intact hemisphere; figure 4.11A). However, this preservation of TH-positive cells in the SNpc following VU0155041 treatment was significantly inhibited following pre-treatment with CPPG (75nmol), whereby levels of TH fell to  $32.3 \pm 1.6\%$  of the intact hemisphere ( $P < 0.01$ , 1-way ANOVA with Dunnett's *post-hoc* test). As anticipated, no significant difference between CPPG and vehicle-treated groups was observed.

Representative nigral sections demonstrating the effects of pre-treatment with CPPG on VU0155041 (100nmol) mediated preservation of TH-positive cells in 6-OHDA lesioned animals are shown in figure 4.11B. The photomicrographs clearly demonstrate supranigral infusion of VU0155041 (100nmol) provided noticeable preservation of TH-positive cells in the lesioned hemisphere. Following pre-treatment with CPPG however, this effect was markedly reduced.

Figure 4.11: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of VU0155041, with respect to tyrosine hydroxylase positive cells in the substantia nigra pars compacta of unilaterally 6-OHDA lesioned rats.

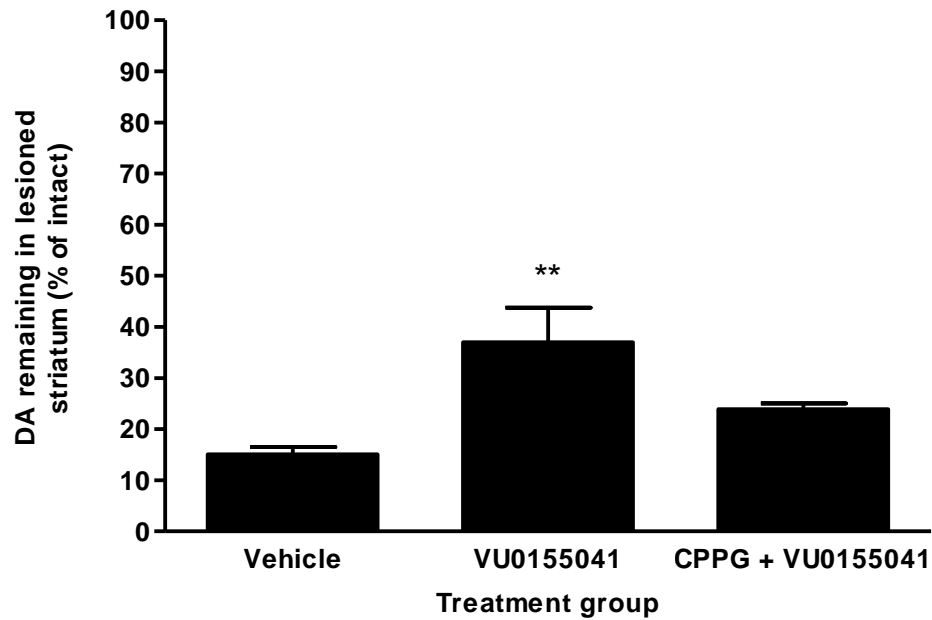


**Figure 4.11.** (A) The effects of sub-chronic supranigral infusion of the broad spectrum antagonist CPPG (75nmol in 4 $\mu$ l of 0.1M NaOH) on the protective effect of VU0155041 (100nmol in 4 $\mu$ l) proceeding 6-OHDA induced loss of TH-positive cells in the SNpc. Values represent mean  $\pm$  s.e.m. (n=7 for all treatment groups). \*\* (P < 0.01) indicates significant difference compared to VU0155041 (100nmol) treated animals. ### (P < 0.001) indicates significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's post-hoc test). F = 17.76; df = 3,23. (B) Representative photomicrographs show the levels of TH immunoreactivity in nigral sections of VU0155041 (100nmol) treated 6-OHDA-lesioned animals following pre-treatment with CPPG. Fixed coronal sections shown here are at -5.30cm AP from bregma, (Paxinos, 1998). Arrow delineates SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **4.4.3.2 Effects of pre-treatment with CPPG on VU0155041 mediated preservation of striatal dopamine content**

VU0155041 (100nmol) mediated preservation of striatal dopamine was also markedly inhibited following pre-treatment with CPPG (75nmol). As expected, the level of protection was significant in absence of CPPG where VU0155041 (100nmol) alone significantly preserved striatal dopamine following a 6-OHDA lesion, amounting to  $37.0 \pm 6.8\%$  of the intact hemisphere with respect to vehicle-treated animals ( $P < 0.01$ , figure 4.12). However, this preservation following pre-treatment with CPPG was no longer significant where striatal dopamine content fell to  $23.9 \pm 1.1\%$  of the intact hemisphere, demonstrating CPPG had blocked this response ( $P > 0.05$ , 1-way ANOVA with Bonferroni's *post-hoc* test). Consistent with previous findings, no significant differences between CPPG and vehicle-treated groups was observed.

Figure 4.12: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of VU0155041, with respect to dopamine content in the striatum of unilaterally 6-OHDA lesioned rats.



**Figure 4.12.** The effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor antagonist CPPG (75nmol in 4 $\mu$ l of 0.1M NaOH) on the protective effect of VU0155041 (100nmol in 4 $\mu$ l), with respect to dopamine content in the striatum of unilaterally 6-OHDA lesioned rats. Values represent mean  $\pm$  s.e.m. (n=7 for all treatment groups). \*\* (P < 0.01) indicates a significant difference compared to vehicle treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). F = 7.356; df = 2,18.



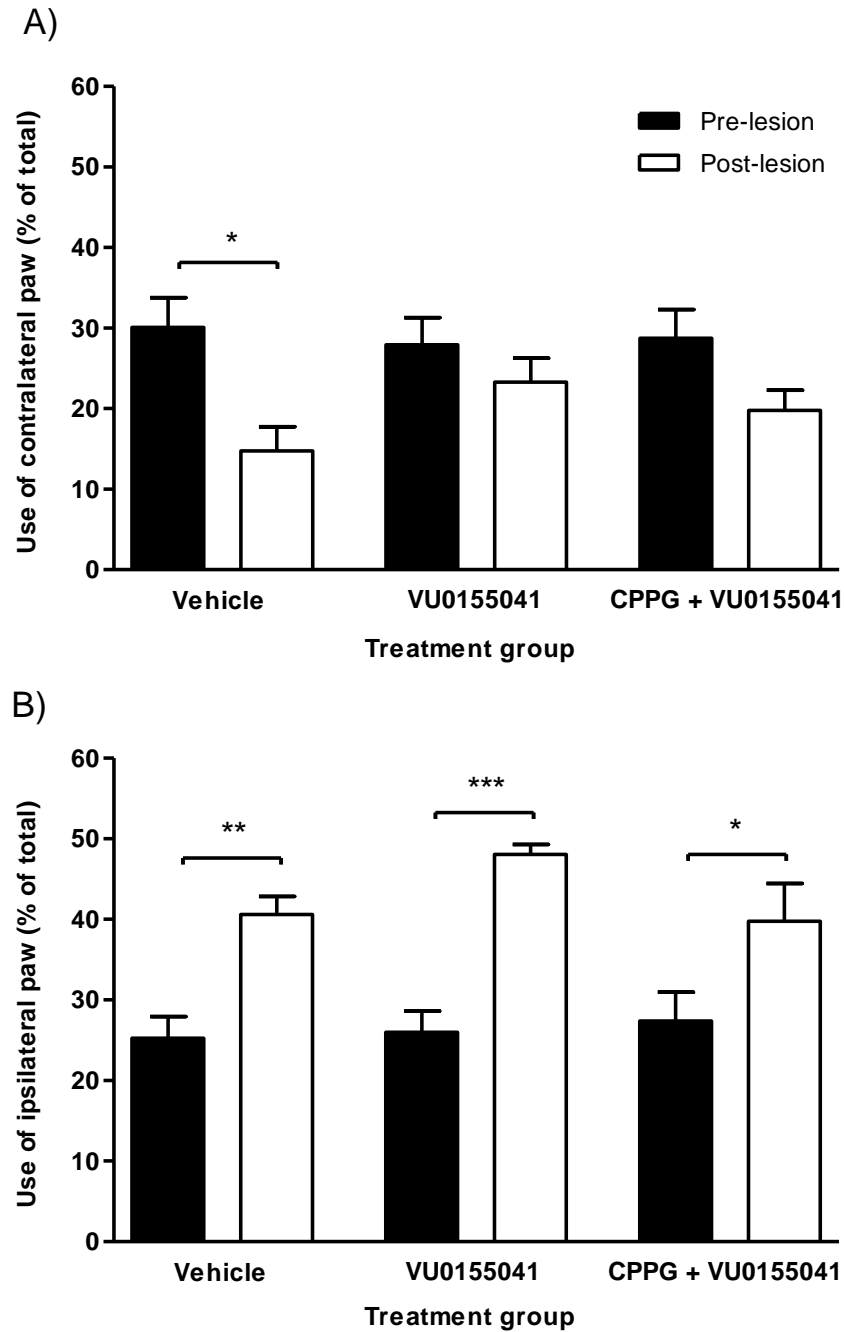
#### **4.4.3.3 Effects of pre-treatment with CPPG on VU0155041 mediated neuroprotection with respect to motor function in 6-OHDA lesioned rats**

##### *Cylinder Test*

The effects of CPPG pre-treatment with VU0155041 (100nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats was assessed using the cylinder test on day 5 post lesion (figure 4.13). As expected in vehicle treated animals, there was a significant decrease in use of the contralateral paw from  $30.1 \pm 3.7\%$  pre-lesion to  $14.7 \pm 3.0\%$  post lesion ( $P < 0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test). Consistent with our previous findings in this chapter, use of the contralateral paw was largely spared with VU155041 (100nmol) following a 6-OHDA lesion (figure 4.13A). Contralateral paw use equated to  $23.3 \pm 3.0\%$  of total paw use post-lesion in comparison to  $27.9 \pm 3.4\%$  pre-lesion, demonstrating approximately 84% preservation in contralateral paw use post-lesion following VU1055041 treatment, which is in very close agreement with our first study in this chapter (85% preservation). Pre-treatment with CPPG (75nmol) reduced the degree of preservation of contralateral paw use with VU0155041 from  $28.7 \pm 3.6\%$  pre-lesion to  $19.8 \pm 2.5\%$  post-lesion, although this effect was not significant ( $P > 0.05$ , 2-way ANOVA with Bonferroni's *post-hoc* test).

Ipsilateral paw use in vehicle-treated animals increased post-lesion by  $>160\%$  with respect to pre-lesion score, compensating for an impaired contralateral paw although to a lesser extent than previously described. As a result, in VU0155041 (100nmol) treated and in CPPG pre-treated animals, use of the ipsilateral paw post-lesion revealed similar post-lesion scores to the vehicle-treated group which was not expected (figure 4.13B).

Figure 4.13: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of VU0155041, with respect to motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



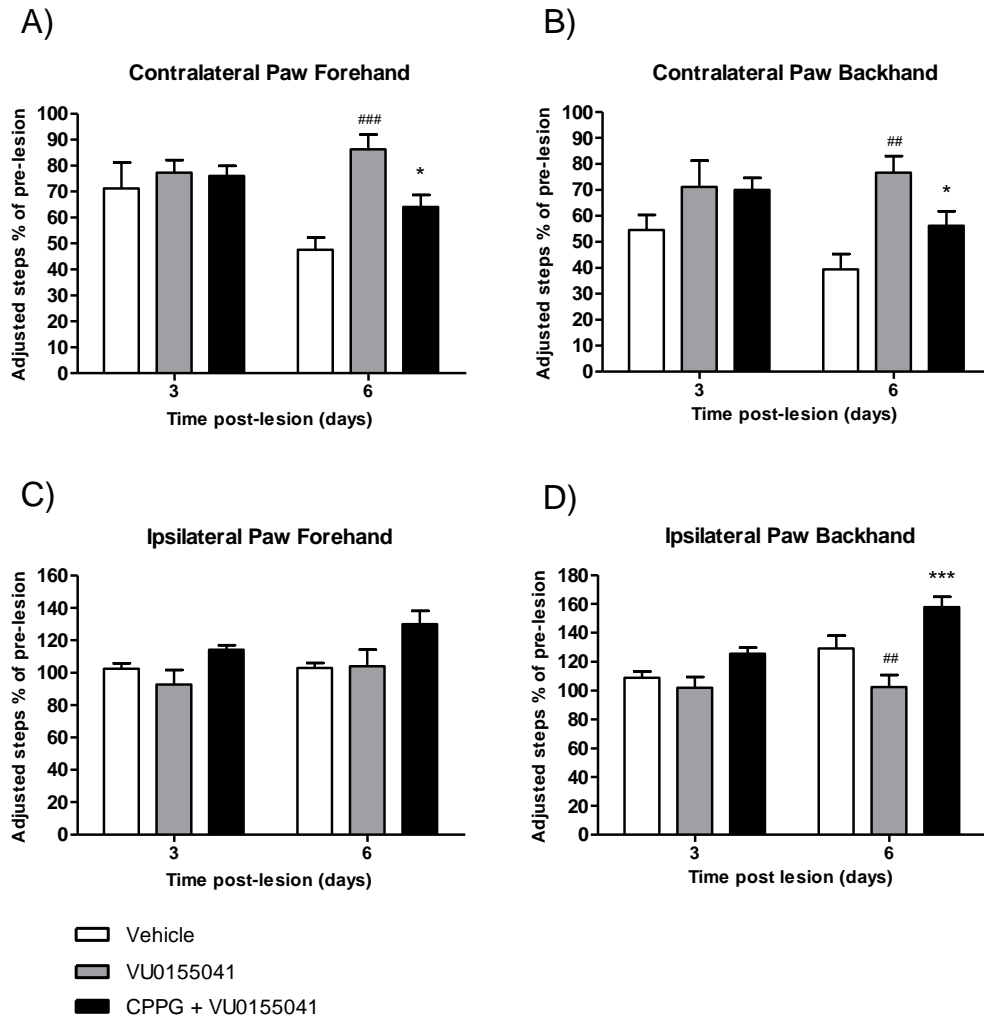
**Figure 4.13.** The effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor antagonist CPPG (75nmol in 4µl of 0.1M NaOH) on the protective effect of VU0155041 (100nmol in 4µl), with respect to (A) contralateral and (B) ipsilateral paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion, expressed as a percentage of total paw use. For all, values represent mean  $\pm$  s.e.m. (n=7 for all treatment groups). \* (P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for pre/post lesion factor, F = 12.48, df = 1,34; for treatment factor F = 0.4066, df = 2,34. In (B) for pre/post lesion factor, F = 42.77, df = 1,34; treatment factor F = 1.019, df = 2,34.

### *Adjusted Stepping*

The effects of CPPG pre-treatment with VU0155041 (100nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 4.14. As expected, the contralateral paw (lesioned) demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $47.6 \pm 4.8\%$  at 6 days post-lesion in the vehicle treated group (figure 4.14A). In this study, VU0155041 (100nmol) treatment significantly preserved contralateral paw use with respect to vehicle-treated animals, equating to  $86.3 \pm 5.6\%$  of pre-lesion scores at 6 days post-lesion ( $P < 0.001$ , 2-way ANOVA with Bonferroni's *post hoc* test). However, following pre-treatment with CPPG, this preservation was significantly reduced to  $64.1 \pm 4.6\%$  of pre-lesion scores at 6 days post-lesion ( $P < 0.05$ ). A similar effect following CPPG pre-treatment was observed in the backhand direction. VU0155041 (100nmol) treatment significantly preserved contralateral paw use with respect to vehicle-treated animals, equating to  $76.7 \pm 6.3\%$  of pre-lesion scores at 6 days post-lesion ( $P < 0.01$ ). However, following pre-treatment with CPPG, this preservation was significantly reduced to  $56.2 \pm 5.6\%$  of pre-lesion scores at 6 days post-lesion ( $P < 0.05$ , figure 4.14B).

As expected the ipsilateral paw in the forehand direction showed no significant deficit in adjusted steps 3 or 6 days post-lesion in vehicle treated animals. However, a significant VU0155041 mediated reduction in adjusted steps as previously reported was seen here in the backhand direction ( $P < 0.01$ , figure 4.14C, D). This effect was totally abolished following pre-treatment with CPPG (75nmol) where post-lesion scores were above those obtained in vehicle treated animals. Collectively these findings demonstrate pre-treatment with CPPG inhibited VU0155041 mediated preservation of motor function shown here by significantly reducing contralateral paw use.

Figure 4.14: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of VU0155041, with respect to motor function assessed using the adjusted steps test in unilaterally 6-OHDA lesioned rats.



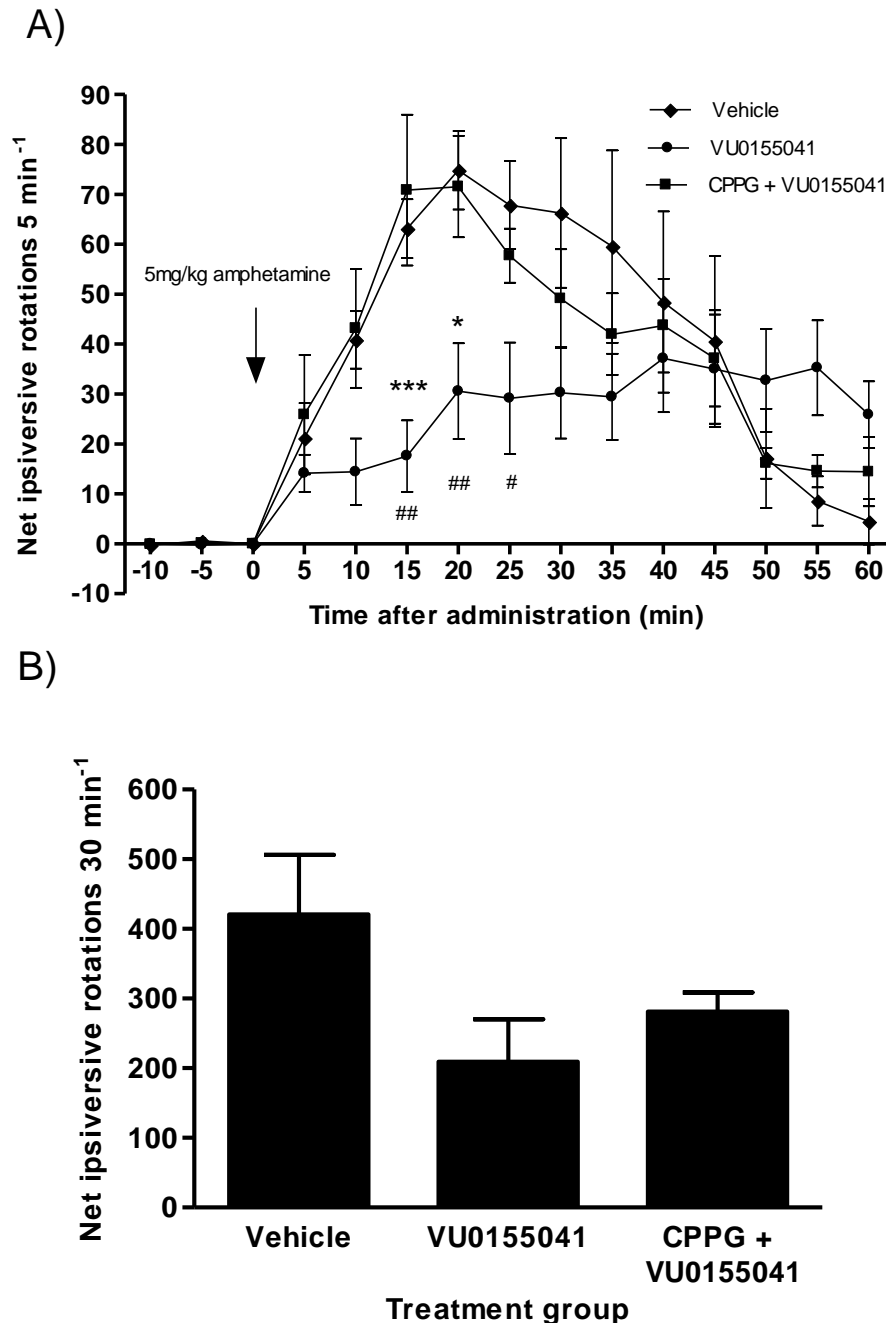
**Figure 4.14.** The effects of sub-chronic supranigral infusion of the broad spectrum group III mGlu receptor antagonist CPPG (75nmol in 4µl of 0.1M NaOH) on the protective effect of VU0155041 (100nmol in 4µl), with respect to contralateral and ipsilateral paw use in the forehand and backhand direction (**A-D**) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion, expressed as a percentage of pre-lesion score. Values represent mean  $\pm$  s.e.m. (n=7 for all treatment groups). \*(P < 0.05) and \*\*\* (P < 0.001) indicates significant differences compared to VU0155041 treated animals. ## (P < 0.01) and ### (P < 0.001) indicates significant differences compared to vehicle-treated animals (both 2-way ANOVA with Bonferroni's *post-hoc* test). In (**A**), for treatment factor, F = 4.765, df = 3, 46 and for days post lesion factor F = 5.116, df = 1, 46. In (**B**), treatment factor, F = 4.588, df = 3, 46 and for days post lesion factor F = 6.034, df = 1, 46. In (**C**), treatment factor, F = 2.859, df = 3, 46 and for days post lesion factor F = 2.531, df = 1, 46. In (**D**), treatment factor, F = 7.985, df = 3, 46 and for days post lesion factor, F = 12.06, df = 1, 46.

### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic VU0155041 following pre-treatment with CPPG are shown in figure 4.15. Analysis of time-course in vehicle treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. As expected, in animals treated with VU0155041 (100nmol) a marked reduction in amphetamine-induced ipsiversive rotations was observed, reaching significance at 15, 20 and 25 min post injection of amphetamine compared to vehicle-treated animals ( $P < 0.01$ , 2-way ANOVA with Bonferroni's *post-hoc* test; figure 4.15A). Pre-treatment of CPPG markedly inhibited VU0155041 mediated reduction in ipsiversive rotations, reaching significance at 15 and 20 min post injection of amphetamine ( $P < 0.001$ , 2-way ANOVA with Bonferroni's *post-hoc* test).

In figure 4.15B, the mean total ipsiversive rotations were quantified over a 30 min (15-45) period to identify the effects of CPPG on VU0155041 mediated reduction in ipsiversive rotations. As expected, VU0155041 (100nmol), markedly decreased amphetamine-induced ipsiversive rotations, shown here, by up to 44% in comparison to vehicle treated animals, however this effect was not significant ( $P > 0.05$ ). Importantly for this study, pre-treatment with CPPG (75nmol), inhibited a VU0155041 mediated reduction in amphetamine-induced rotations where the number of rotations equated to  $281 \pm 28$  in  $30\text{ min}^{-1}$  in comparison to  $209 \pm 61$   $30\text{ min}^{-1}$  in VU0155041 treated animals. Again however, this change was not significant.

Figure 4.15: Effects of the group III mGlu receptor antagonist CPPG on the protective effect of VU0155041, with respect to motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 4.15.** Effects of the group III mGlu receptor antagonist CPPG (75nmol in 4 $\mu$ l of 0.1M NaOH) on the protective effect of VU0155041 (100nmol in 4 $\mu$ l), with respect to amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. (A) Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle, VU0155041 and CPPG pre-treated animals. (A) \* (P<0.05), \*\*\* (P<0.001) indicate significance compared to CPPG pre-treated animals. # (P<0.05), ## (P<0.01) indicate significant difference compared to vehicle-treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). For treatment factor, F = 7.937, df = 2, 270 and for time, F = 15.06, df = 14, 270. (B) Total ipsiversive rotations induced by amphetamine in 30 min<sup>-1</sup> in vehicle, VU0155041 and CPPG pre-treated animals. F = 2.933, df = 2, 18. Values represent mean  $\pm$  s.e.m. (n=7 for all treatment groups).

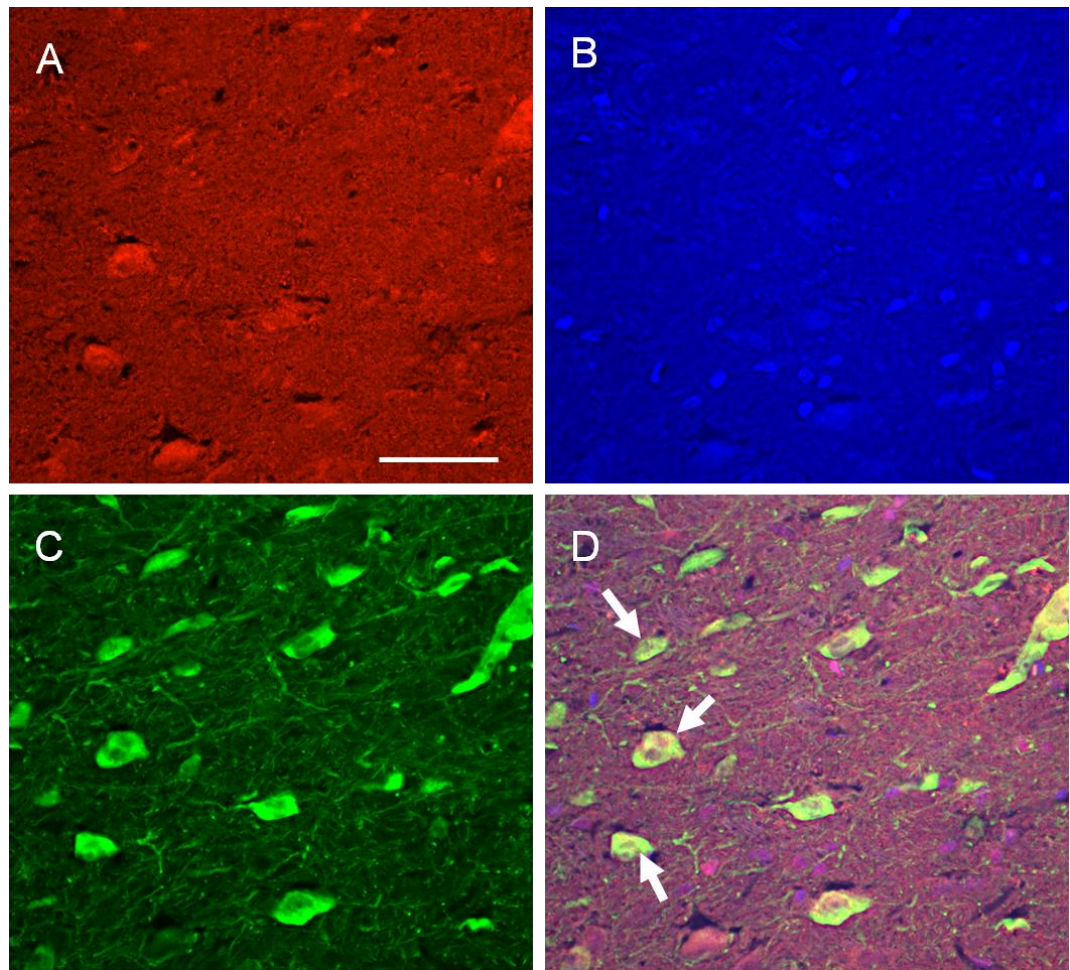
#### **4.4.4 Preliminary study to determine localisation of mGlu4 receptors on dopaminergic neurons and astrocytes in the SNpc of naïve rats**

##### **4.4.4.1 Co-localisation of mGlu4 receptors with tyrosine hydroxylase**

This final study set out to investigate whether mGlu4 receptors were co-localised on dopaminergic neurons in the SNpc of naïve rat brain using fluorescence microscopy. The results from a representative set of sections in the SNpc are shown in figure 4.16.

Assessment of mGlu4 receptors alone revealed diffuse homogenous staining throughout the SNpc consistent with previous findings in Chapter 2, suggesting a likely pre-synaptic axon terminal location. Interestingly, shown here, immunoreactivity to mGlu4 was punctate, indicating some mGlu4 receptors in the SNpc may also be post-synaptic in origin (figure 4.16A). This punctate staining for mGlu4 was not co-localised with resident nuclei demonstrating no overlap with the nuclear stain Hoechst, implying antibody binding was specific to mGlu4 receptors (figure 4.16B). Analysis of TH in the same region of the SNpc, as expected, revealed wide distribution of TH-positive cells throughout (figure 4.16C). On merging all three images, a number but not all of the presumed post-synaptic mGlu4 receptors were shown to be co-localised with TH positive cells (figure 4.16D). This would suggest post-synaptic mGlu4 receptors are localised on dopaminergic neurons within the SNpc.

Figure 4.16: Co-localisation of mGlu4 receptors with tyrosine hydroxylase in the SNpc of naïve rat brain.



**Figure 4.16.** Photomicrographs of immunofluorescence for (A) mGlu4 receptors [red], (B) nuclei (Hoechst) [blue], (C) TH-positive cells [green] and (D) merged image of all three markers in a region of the SNpc in naïve rat brain. In D, white arrows indicate co-localisation of mGlu4 receptors with TH-positive cells. Scale bar is equal to 50 $\mu$ m.

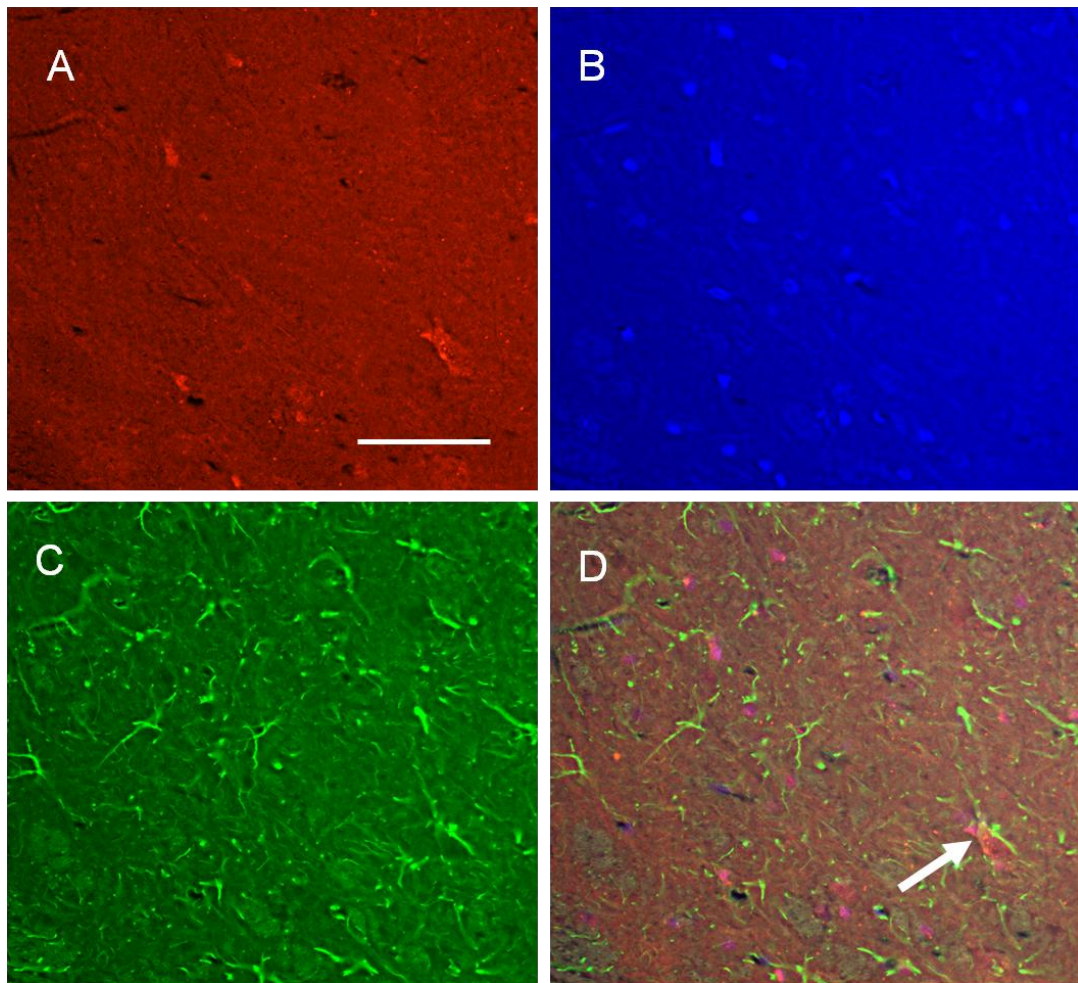


#### **4.4.4.2 Co-localisation of mGlu4 receptors with glial fibrillary acidic protein**

Immunofluorescence microscopy was utilised to investigate co-localisation of mGlu4 receptors on astrocytes in the SNpc of naïve rat brain. The results from a representative set of sections in the SNpc are shown in figure 4.17.

Immunoreactivity for mGlu4 receptors in the SNpc again appeared predominantly of a diffuse fibrous nature consistent with the notion of a pre-synaptic axon terminal location. In addition, mGlu4 immunoreactivity was also punctate, suggesting mGlu4 receptors may also reside post-synaptically in the SNpc as previously described (figure 4.17A). The punctate staining for mGlu4 largely appeared not to be co-localised with resident nuclei using the nuclear stain Hoechst. However, on closer visual inspection, co-localisation was observed, suggesting a degree of non-specific binding with this antibody (figure 4.16B). Staining for GFAP in the same region of the SNpc, revealed wide distribution of GFAP-positive cells demonstrating morphology reminiscent of both quiescent and activated (star-shaped characteristic) astrocytes (figure 4.17C). On merging all three images, very little co-localisation of mGlu4 receptors with GFAP-positive cells was observed in the SNpc. However in one instance in figure 4.16D, co-localisation of mGlu4 with GFAP was clearly apparent. These findings suggest post-synaptic mGlu4 receptors may be localised on astrocytes, however likely account for a minority of mGlu4 receptors in the SNpc. Thus, owing to the limited co-localisation observed here, post-synaptic mGlu4 receptors in the SNpc are unlikely to reside on astrocytes.

Figure 4.17: Co-localisation of mGlu4 receptors with glial fibrillary acidic protein in the SNpc of naïve rat brain.



**Figure 4.17.** Photomicrographs of immunofluorescence for (A) mGlu4 receptors [red], (B) nuclei (Hoechst) [blue], (C) GFAP-positive cells [green] and (D) merged image of all three markers in a region of the SNpc in naïve rat brain. In D, white arrow indicates co-localisation of mGlu4 receptor with GFAP-positive cell. Scale bar is equal to 50 $\mu$ m.

## 4.4 Discussion

In this chapter we demonstrate for the first time, using the selective positive allosteric modulator (PAM) VU0155041, that mGlu4 receptor activation provides functional neuroprotection in the 6-OHDA rat model of PD. In addition, the effects of VU0155041 were inhibited by pre-treatment with the group III mGlu receptor antagonist CPPG, confirming responses were mediated by activation of the mGlu4 receptor. Furthermore, protection mediated by VU0155041 was accompanied by a reduction in inflammatory markers, suggesting that a reduced inflammatory response may contribute in part to this neuroprotective effect.

The studies presented in this chapter, demonstrate the mGlu4 selective PAM VU0155041 administered 1h before and for 7 days post a 6-OHDA lesion significantly protected the nigrostriatal tract. This was evidenced by the dose-dependent preservation of TH-positive cells in the SNpc and reduction in 6-OHDA-induced loss of striatal TH and DDC immunoreactivity. These preserved markers of nigrostriatal tract integrity were reflected in elevated dopamine content in both the SN and striatum of VU0155041 versus vehicle-treated animals, as measured by HPLC. This finding is consistent with those of Battaglia *et al.*, (2006), who found that systemic injections of the mGlu4 PAM PHCCC, given 30 min before toxin injection, offered approximately 50% protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of striatal dopamine content and reduction in TH-positive cell numbers in the SNc in mice.

In addition to confirming a neuroprotective effect at the neurochemical and immunohistochemical level following treatment with the mGlu4 PAM VU0155041, we demonstrate here protection led to a preservation of motor function following a unilateral injection of 6-OHDA. Significant preservation of contralateral paw use was shown in both the cylinder and adjusted stepping test following VU0155041 treatment in addition to a reduction in amphetamine-induced ipsiversive rotations reflecting reduced asymmetry between the lesion and intact hemispheres in this hemiparkinsonian model. Furthermore in a separate study, treatment with the broad spectrum antagonist CPPG prior to VU0155041 markedly attenuated both the

behavioural end-points and the post-mortem indices of preserved nigrostriatal tract function, confirming VU0155041 produced these effects via a receptor mediated process. In certain instances the effects of CPPG to inhibit responses at the behavioural level were not significant therefore it remains to be determined whether higher doses of CPPG would have inhibited the residual responses of VU0155041.

In the initial dose finding study, preservation of dopamine content in both the striatum and SN (~70% of intact hemisphere) following treatment with VU0155041 was greater than that in the follow-up study investigating preservation of TH (~45%). These contrasting levels of protection are likely due to the variability in lesion size, where in the initial study only a ~70% lesion in the SN was obtained compared to the desired ~90% in the full study. Therefore the larger investigation offers a more valid assessment of the neuroprotective potential of mGlu4 receptor activation in this experimental paradigm.

The degree of protection seen with VU0155041 according to levels of TH in the SN (45% preservation) was slightly less than that achieved in the previous chapter using the broad spectrum agonist L-AP4 (~60% preservation of TH). Nevertheless, this indicates that a large proportion of L-AP4's effects were likely attributable to mGlu4 receptor activation. The maximal dose of VU0155041 tested here (100 nmol) was based on that used i.c.v. by Niswender *et al* (2008) against haloperidol-induced catalepsy and reserpine-induced akinesia. However, a recent study has shown doses of 500 nmol can be tolerated intrathecally (Wang *et al.*, 2011), paving the way for examination of higher doses in the future that may afford a greater degree of protection, akin to that seen with L-AP4. The lack of comparable protection with L-AP4 seen here with VU0155041 may also suggest a contribution from activation of additional group III mGlu receptors, a possibility explored in the next chapter of this thesis. The protective effects of VU0155041 are certainly consistent with the intense staining for mGlu4 in the SNpc described in Chapter 2 of this thesis. Some of this immunoreactivity most likely represents mGlu4 receptors located on excitatory (glutamatergic) terminals, as has been specifically demonstrated in the neighbouring SNpc (Corti *et al.*, 2002; Kosinski *et al.*, 1999). However, the precise anatomical

location of mGlu4 receptors on other neurones or additional cell types in this region remains to be examined.

Interestingly, previous reports using group III mGlu receptor broad spectrum agonists in electrophysiological responses *in-vitro*, through to the behavioural and neuroprotective findings with L-AP4 *in-vivo* discussed in chapter 3, have shown effects tail off at the higher concentrations or doses tested (MacInnes *et al.*, 2004; Valenti *et al.*, 2003; Vernon *et al.*, 2007b) thought to be a result of receptor desensitisation/ internalisation. This effect was not apparent in our studies using VU0155041 suggesting targeting the allosteric site of the receptor may be an effective strategy to reduce the likelihood of receptor desensitisation (Kew, 2004). Indeed, pharmacological modulation of an allosteric site over a highly conserved orthosteric binding site may offer numerous advantages in addition to a lack of desensitisation and internalization, such as higher subtype selectivity and lack of downregulation along with reduced side effects (Wood *et al.*, 2011). Taken together, these facets likely explain the recent emphasis on developing further mGlu4 receptor PAMs (East *et al.*, 2010; Engers *et al.*, 2010; Niswender *et al.*, 2008b; Williams *et al.*, 2009) to probe the functional involvement of these receptors.

Owing to the positive effect of VU0155041 in these studies, it is pertinent at this stage to consider potential mechanisms underlying this protection. Whilst numerous mechanisms have been proposed to underlie mGlu4 receptor-mediated neuroprotection (reviewed in Duty, 2010), the two most likely mechanisms include inhibition of glutamate release in the SNpc to facilitate protection from glutamate-mediated excitotoxicity, or reduced inflammatory actions. Previous data from this and other laboratories already provides some support for the glutamate hypothesis, while the findings presented in this chapter has provided new evidence in support of a potential anti-inflammatory contribution.

In relation to the glutamate hypothesis, electrophysiological findings have shown the broad spectrum agonist L-AP4 depresses STN-evoked EPSCs in the SNpr (Wittmann *et al.*, 2001; Wittmann *et al.*, 2002), and inhibits EPSPs in dopaminergic neurones of the SNpc (Wigmore & Lacey, 1998), most likely reflecting inhibition of glutamate

release from STN terminals in these regions. In support of this, we found that L-SOP and L-AP4 inhibited depolarisation-evoked release of [<sup>3</sup>H]-D-aspartate from slices of rat SN *in-vitro* and that local intranigral infusion of L-SOP reduced glutamate release in the SNpr *in- vivo* (Austin *et al.*, 2010). Furthermore mGlu4 receptors have been identified as key functional subtypes involved, since STN-evoked EPSCs in dopaminergic neurones of the SNpc were inhibited by the mGlu4 PAM, PHCCC (Valenti *et al.*, 2005). Indeed, this is consistent with findings in our laboratory that show L-SOP and L-AP4 inhibit depolarisation-evoked release of [<sup>3</sup>H]-D-aspartate from slices of rat SN *in-vitro* while local intranigral infusion of L-SOP reduces glutamate release in the SNpr *in- vivo* (Austin *et al.*, 2010). These effects of L-AP4 *in vitro* were potentiated by PHCCC which further supports involvement of mGlu4 receptors in restricting glutamate release in the SN in general (Broadstock *et al.*, 2011). Further microdialysis studies would be of real worth here to support the notion that inhibition of glutamate release in the SNpc contributes to the neuroprotective effects following mGlu4 receptor potentiation. In the MPTP study of Battaglia *et al.*, (2006), protection against MPTP-induced degeneration was seen following injection of PHCCC directly into the globus pallidus which lies upstream of the SNpc in the indirect basal ganglia circuit. This finding implies that mGlu4 receptor-mediated normalisation of glutamatergic drive to the SNpc, either directly by actions in the SNpc itself or indirectly via correction of pallidosubthalamic firing and subsequent downstream reductions in STN glutamatergic drive to the SNpc, might provide effective neuroprotection in these toxin-induced animal models of PD.

The presence of mGlu4 receptors on both astrocytes and microglia in culture (Taylor *et al.*, 2003; Yao *et al.*, 2005), raises the possibility that a further glial component may contribute to the overall protective actions of mGlu4 modulators. Certainly, inflammation is believed to play a key role in PD pathogenesis (Gerhard *et al.*, 2006; Mogi *et al.*, 2000; Tansey & Goldberg, 2010; Whitton, 2007) and microglial activation and elevated inflammatory markers are similarly seen in the striatum and SNpc of rats following a 6-OHDA lesion (Cicchetti *et al.*, 2002; Mogi *et al.*, 2000). It is thought that following a pathologic stimulus of this kind, substantial increases in pro-inflammatory cytokines and cell adhesion molecules occur, whereupon the activated microglia cluster around dopaminergic neurones and become phagocytic

(Banati *et al.*, 1998; Bronstein *et al.*, 1995). The progressive degeneration is worsened by the release of chemo-attractants from the dying neurones which leads to further activated microglia infiltrating the region. L-AP4 treatment of astrocytes has been shown to reduce the production of such pro-inflammatory chemokines (Besong *et al.*, 2002) and more importantly, this effect was abolished in astrocyte cultures from mGlu4 *-/-* mice, implicating astroglial mGlu4 receptors in mediating potential anti-inflammatory actions. The marked decrease in GFAP-positive cells observed here following VU0155041 treatment is certainly supportive of a role for mGlu4 receptor activation in restricting 6-OHDA-induced astroglial-driven neurotoxicity *in vivo*. Treatment with the mGlu4 PAM also lead to significant reduction in activation of microglia in the SNpc of 6-OHDA rats, as shown by the reduced levels of IBA-1 in the lesioned hemisphere of VU0155041-treated animals compared to that of vehicle treated animals.

Whilst the presence of mGlu4 receptors has been shown on astrocytes and microglia in culture, despite much speculation, no study to our knowledge has confirmed such a presence in the rodent brain. To investigate this possibility, a final co-localisation study was performed to determine the presence of mGlu4 receptors on astrocytes in the rat SNpc. Interestingly very little co-localisation of mGlu4 with GFAP was observed in the SNpc suggesting mGlu4 receptors are in fact unlikely to reside on astrocytes. Whilst localisation of mGlu4 receptors on microglia has not been investigated, these findings would suggest the anti-inflammatory effects of VU0155041 most likely reflect an indirect effect via stimulation of neuronal mGlu4 receptors. However further studies particularly *in vivo*, are no doubt required to elucidate the contribution of an anti-inflammatory action in mediating neuroprotective effects. These preliminary studies also revealed a degree of co-localisation of likely post-synaptic mGlu4 receptors on dopaminergic neurones (shown by TH-positive cells), raising the possibility of a post-synaptic action of mGlu4 receptors in mediating neuroprotection. Currently however, there is little evidence in favour of a post-synaptic action of mGlu4 receptors in the SNpc. Indeed, Wigmore & Lacey (1998) failed to detect any direct post-synaptic effects of L-AP4 on SNpc neurones however mGlu4 selective agents were not investigated. Therefore additional studies are required to clarify whether mGlu4 receptors may mediate any post-

synaptic action, distinct from modulation of neuronal excitability which may relate to a potential neuroprotective effect.

Nonetheless, the notion that multiple mechanisms are involved in mediating functional neuroprotection may explain why considerably lower doses of VU0155041 (100nmol) are required in the 6-OHDA model compared to that required to produce reversal of reserpine-induced akinesia (316nmol) which largely relies on inhibition of glutamate release alone (Niswender *et al.*, 2008). However, despite little change in receptor expression following a 6-OHDA lesion (Messenger *et al.*, 2002), we cannot rule out a post-lesion increase in sensitivity of these receptors which could also underlie the lower doses of VU0155041 required in this model of PD.

To conclude, the studies reported here demonstrate selective activation of mGlu4 receptors using the selective PAM V0155041 can provide functional neuroprotection in the 6-OHDA rat model of PD. The notion that several mechanisms, including restricting glutamate release and provision of an anti-inflammatory component, may be involved in mediating this protection is particularly appealing given the likelihood that multiple pathogenic events lead to PD. It is hoped by implementing multiple mechanisms, mGlu4 receptor activation may offer improved translation in additional animal models of PD as a neuroprotective or disease modifying strategy which has in the past so often failed when targeting one mechanism alone (as described in Duty & Jenner, 2011). Finally, with the degree of protection seen with VU0155041 being slightly less than that achieved with the broad spectrum agonist L-AP4, it is encouraging to suggest the additional group III mGlu receptor subtypes mGlu7 and 8 may also mediate functional neuroprotection in the 6-OHDA rat model of PD.



**Chapter 5:** Potential of targeting metabotropic glutamate receptor 7 and 8 to provide functional neuroprotection in the 6-OHDA rat model of PD

## 5.1 Introduction

In chapter 4 of this thesis we demonstrated that activation of mGlu4 receptors using the selective positive allosteric modulator VU0155041 significantly protects against 6-OHDA-induced nigrostriatal tract degeneration in rats and mediates preservation of motor behaviour in these animals. These findings confirmed for the first time that neuroprotection could be mediated exclusively upon activation of a single group III mGlu receptor subtype in the 6-OHDA rat model of PD. In this chapter we seek to determine the ability of mGlu7 and mGlu8 receptors to similarly provide functional neuroprotection in the 6-OHDA rat model of PD.

Assessment of group III mGlu receptor distribution, in Chapter 2 of this thesis revealed high intensity staining for mGlu7 receptors in the dorsal tier of the SNpc, SNpr and STN and moderate intensity staining in the striatum and GP (internal and external segments). Indeed additional studies have confirmed the presence of mGlu7 receptors in numerous basal ganglia nuclei demonstrating particularly high expression in the striatum and the SN (Bradley *et al.*, 1999; Kosinski *et al.*, 1999). Furthermore in the striatum, mGlu7 receptors have been shown to be localized presynaptically on the corticostriatal glutamatergic synapses, where they are found to decrease the glutamatergic tone and attenuate transmission in cholinergic interneurons (Bonsi *et al.*, 2007; Pisani *et al.*, 1997). Thus mGlu7's wide distribution in the basal ganglia circuit suggests that it may display a fundamental role in the control of normal and abnormal motor activity (Kinoshita *et al.*, 1996; Kosinski *et al.*, 1999). In particular, expression of mGlu7 receptors in the subthalamonigral terminal regions would suggest these group III mGlu receptor subtypes could hold promise as potential candidates to mediate functional neuroprotection.

There is limited evidence to date to supporting a neuroprotective role of mGlu7 receptors. However, early *in vitro* studies which showed activation of group III mGlu receptors using L-AP4 could protect against NMDA-induced toxicity in mixed cultures of mouse cerebellar granule neurones offers some support. This stems from the fact that high concentrations of L-AP4 (1-3mM) used in these studies would suggest mGlu7 may mediate this effect since the EC<sub>50</sub> of L-AP4 at mGlu7 is greater

than 100 $\mu$ M, 100-fold higher than at mGlu4 or 8 (Lafon-Cazal *et al.*, 1999a). Furthermore this effect was not apparent at lower concentrations of L-AP4 supporting a lack of involvement of mGlu4 and mGlu8 receptors.

Progress in elucidating a neuroprotective role for mGlu7 receptors in PD has been hampered by a lack of selective pharmacological tools. However the relatively recent discovery of an orally active, brain-penetrable and selective allosteric activator of mGlu7 receptors, N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082) (Mitsukawa *et al.*, 2005), has opened up the possibility in establishing a role for mGlu7 in PD. AMN082 directly activates receptor signalling via an allosteric site in the transmembrane domain demonstrating little effect on the potency of orthosteric ligands. In transfected mammalian cells expressing mGlu7, AMN082 potently inhibits cAMP accumulation and stimulates GTP $\gamma$ S binding (EC<sub>50</sub>-values, 64-290nM; Mitsukawa *et al.*, 2005) with agonist efficacies comparable to those of L-AP4 and superior to those of glutamate (EC<sub>50</sub> values are documented in Table 3.1). Selectivity profiling of AMN082 assessed using the GTP $\gamma$ S binding radioligand displacement assay in CHO cells revealed no activating or inhibitory effect at other mGlu receptor subtypes and selected iGlu receptors with concentrations less than or equal to 10 $\mu$ M (Mitsukawa *et al.*, 2005). Furthermore no significant binding interaction of 1 $\mu$ M AMN082 with 30 additional nervous system targets including receptors for adrenaline, dopamine, GABA, histamine, acetylcholine, opiates, serotonin and substance P was reported in this initial study. However the specific receptor subtypes of these transmitter systems examined in these studies are not described so activity at all subtypes cannot categorically be ruled out.

Following the discovery of AMN082, a recent study has attempted to examine the antiparkinsonian effects following mGlu7 receptor activation *in vivo*. Intrastratial administration (0.1 and 0.5nmol) of AMN082 was shown to reverse haloperidol-induced catalepsy in rats. Furthermore this reduction in catalepsy was not apparent in mGlu7 receptor knockout mice suggesting that these antiparkinsonian effects could be mediated by activation of (assumed pres-synaptic) mGlu7 receptors on the glutamatergic corticostriatal synapses (Greco *et al.*, 2010). This study also went on to show AMN082 (2.5 and 5mg/kg i.p.) reduced apomorphine-induced rotations in

unilateral 6-OHDA lesioned rats and in a more complex task commonly used to evaluate major akinetic symptoms of PD patients, AMN082 (5mg/kg) was shown to reverse the increased reaction time to respond to a cue in bilateral 6-OHDA lesioned rats. The low doses used for intrastriatal administration of AMN082 in these studies (0.1-0.5nmol) are likely an attempt to adhere to the narrow window of selectivity initially reported for AMN082 at mGlu7 ( $\leq 10\mu\text{M}$ , Mitsukawa *et al.*, 2005). Furthermore, studies in this laboratory have recently demonstrated intranigral administration of AMN082 is also able to reverse parkinsonian-like symptoms in the reserpine-treated rat model (Broadstock *et al.*, 2011). Although these effects were presumed to reflect inhibition of glutamate release following activation of mGlu7 receptors on subthalamonigral terminals, this mechanism of action remains to be confirmed. However, in this same study AMN082 was shown to significantly inhibit KCl-evoked [ $^3\text{H}$ ]-D-aspartate release in nigral slices *in vitro* (Broadstock *et al.*, 2011) suggesting activation of mGlu7 receptors could mediate this desired outcome. Therefore, these findings point to a strong likelihood that activation of mGlu7 receptors in the SNpc, may well provide some degree of neuroprotection.

As for assessing the potential of targeting mGlu8, distribution studies described in chapter 2 of this thesis revealed widespread expression throughout the basal ganglia although to a much lesser degree than that seen for both mGlu4 and 7. This pattern is in agreement with in-situ hybridisation studies performed previously in our laboratory, which reported particularly low levels of mGlu8 mRNA in the GPe, GPi and the SNpr (Messenger *et al.*, 2002). The distribution of mGlu8 receptors in the basal ganglia is the least well characterised of all the group III mGlu receptor subtypes, therefore no additional studies supplement these preliminary findings. However, expression of mRNA encoding mGlu8 receptors has been reported in the cortex, striatum and STN (Messenger *et al.*, 2002), suggesting the possibility that mGlu8 receptors are found on pre-synaptic terminals of corticostriatal, striatonigral and subthalamonigral pathways. The presence particularly in the subthalamonigral pathway would favour a potential role for mGlu8 receptors to provide neuroprotection in our experimental paradigm.

Autoradiographic studies also offer some support for the presence of mGlu8 in the basal ganglia. Visualisation of group III mGlu receptors, using [<sup>3</sup>H]-L-AP4, has shown high levels of binding within the globus pallidus (GP) and SNpr, moderate levels in the striatum, and low levels within the STN and SNpc in rat brain (Hudtloff & Thomsen, 1998). Furthermore, studies comparing [<sup>3</sup>H]-L-AP4 (30nM) binding in wild-type and mGlu4 knock-out mice revealed 28% of specific binding remains in the SNpr of the knock-out mice (Thomsen & Hampson, 1999). Although these results confirm a significant proportion of L-AP4 binding is due to mGlu4 in this region, the remaining high-affinity specific binding could be due to mGlu8 binding, rather than mGlu7 which requires, as noted above higher (μM range) concentrations for activation (Cartmell & Schoepp, 2000). However no mGlu8 knock-out studies have been used to confirm this notion.

As previously mentioned, very few selective pharmacological agents have been identified for group III mGlu receptors. Currently, the mGlu8 receptor agonist (*S*)-3,4-dicarboxyphenylglycine (DCPG) remains the only selective pharmacological tool to probe the involvement of mGlu8 receptors. Initially described by Thomas *et al.*, (2001), (*S*)-3,4-DCPG has been characterised on cloned human mGlu1–8 receptors individually expressed in AV12-664 cells co-expressing a rat glutamate/aspartate transporter. Data from the assays on each of the eight mGlu receptor subtypes demonstrated (*S*)-3,4-DCPG is at least 100-fold more selective at mGlu8 (EC<sub>50</sub> of 31nM) than additional mGlu receptor subtypes (EC<sub>50</sub> values >3.5 mM on mGlu1–7). This study further identified the (*S*)-isomer to be more potent and selective than the (*R*)-isomer of 3,4-DCPG which demonstrated antagonist activity at AMPA receptors on rat motor neurons (Thomas *et al.*, 2001). Thus the (*S*)-isomer is favoured to probe involvement of mGlu8 receptors and all further reference to DCPG in this thesis refers to this isomer.

To determine the ability of mGlu8 to inhibit glutamate release *in vitro*, previous findings in our laboratory have shown selective activation of mGlu8 receptors using DCPG over a wide concentration range (0.03 – 10 μM) spanning the estimated EC<sub>50</sub> of 31 nM (Thomas *et al.*, 2001), had no significant effects on either basal or 25mM KCl-evoked [<sup>3</sup>H]-D-aspartate release in nigral slices *in vitro* (Broadstock *et al.*,

2011). This would suggest mGlu8 receptors may well be less prominent in inhibiting glutamate release from overactive glutamatergic projections in the basal ganglia in a rat model of PD. Nonetheless, an interesting observation from the neuroprotection studies using VU0155041 in Chapter 3 of this thesis, is that the level of protection achieved following mGlu4 receptor activation was below that seen with the broad spectrum agonist L-AP4. For example in the striatum, preservation of TH equated to ~45% of the intact hemisphere following treatment with VU0155041, yet > 63% of TH was preserved in L-AP4 treated rats following a 6-OHDA lesion. Whilst these findings would suggest mGlu4 receptors are largely responsible for the protection observed with L-AP4, the additional protective effect may arise from other group III mGlu receptor subtypes, namely mGlu7 or mGlu8. Based on the literature described thus far, mGlu7 is likely involved in this residual response, however a role for mGlu8 cannot be ruled out.

The studies described in this chapter seek to further our quest in elucidating the potential of targeting group III mGlu receptors as an alternative, non-dopaminergic, therapy to provide neuroprotection in the treatment of PD. In particular the main objectives to be addressed in this chapter was to examine the potential of targeting mGlu7 and mGlu8 receptors using the respective selective ligands AMN082 and DCPG to provide functional neuroprotection in the 6-OHDA rat model of PD. Although both these agents are thought to demonstrate a degree of systemic activity, the studies described here seek to assess the efficacy of both agents following site-directed injections directly into the SNpc using the same experimental paradigm described in the previous chapter. Through direct injection of agents into the SNpc, we can be sure that any effects seen are the result of targeting receptors in this region alone; such information is valuable not only in teasing out the role of receptors within this region, but also in predicting the likely mechanisms underpinning any observed response.

## 5.2 Aims

The overall aim of the studies described in this chapter was to investigate whether activation of mGlu7 or mGlu8 receptors provides functional neuroprotection in the 6-OHDA rat model of PD.

More specifically the key objectives here were:

1. to determine if the mGlu7 selective positive allosteric agonist AMN082 and mGlu8 selective agonist DCPG provide neuroprotective effects against a 6-OHDA lesion of the SNpc in rats, determined by immunohistochemical and neurochemical analysis
2. to ascertain whether any neuroprotective effects of AMN082 and DCPG translate to functional restoration of motor function as assessed in both forced and habitual behavioural tasks
3. to establish the effective dose range of AMN082 and DCPG to provide neuroprotection as determined by behavioural and immunohistochemical assessment
4. to investigate whether AMN082 or DCPG treatment leads to astrocytic and microglial activation as a potential neuroprotective mechanism

## **5.3 Methods**

### **5.3.1 Specific methodological details for AMN082 and DCPG neuroprotection studies**

#### **5.3.1.1 Animals**

Male Sprague Dawley rats (B & K or Harlan, U.K.) weighing 270 – 300 g were used in these studies. Food and water were provided *ad libitum*. Animals were housed in a temperature- and humidity-controlled environment with a 12-h light / dark cycle. All procedures conformed to the U.K. Animals (Scientific Procedures) Act, 1986 and every effort was made to minimise animal numbers and suffering.

#### **5.3.1.2 Surgical cannulation**

Animals were bilaterally cannulated in the same manner as previously described in section 3.3.2.4 whereby cannulae were implanted bilaterally 2mm above the SNpc using the following co-ordinates; anteroposterior (AP), -4.8mm, mediolateral (ML),  $\pm 2.0$ mm; dorsoventral (DV), -6.3mm from bregma (Paxinos & Watson, 1998).

#### **5.3.1.3 Supranigral drug administration**

A minimum of 5 days following cannulae implantation, the neuroprotection study commenced. Animals received their first dose of AMN082, DCPG or vehicle 1 hour prior to 6-OHDA infusion (detailed in 5.3.1.4). AMN082 was administered at doses of 0.5, 1, 10 and 100nmol, dissolved in 4 $\mu$ l PBS with 10% DMSO, while DCPG was administered at 1, 10 and 30nmol in 4 $\mu$ l PBS. Animals received unilateral drug infusions at a flow rate of 2 $\mu$ l min<sup>-1</sup> into the right cannulae (or left on occasions when the right was blocked) for a further 7 days post-lesion as previously described in section 3.3.2.3.



#### **5.3.1.4 Intranigral 6-OHDA lesioning**

A minimum of 5 days following bilateral cannulation above the SNpc, animals received a single, unilateral injection of 6-OHDA into the same side of the SNpc as the drug infusions. 30 min prior to 6-OHDA injection, rats were pre-treated with desipramine (25 mg kg<sup>-1</sup> i.p.) and pargyline (5 mg kg<sup>-1</sup> i.p.). Animals were then anaesthetised briefly whilst 6-OHDA (12 µg in 2.5 µl of 0.2% ascorbic acid in 0.9% saline) was injected at a rate of 1.25 µl min<sup>-1</sup> into the SNpc (2 mm below the guide cannula) as previously described in section 3.3.2.4. Animals were placed in cages on thermostatically heated mats until fully conscious.

#### **5.3.1.5 Behavioural assessment**

The extent of motor impairment following a unilateral lesion to the SNpc was assessed at intervals using three behavioural tests. Animal behaviour was assessed using the adjusted steps test on day 3 and 6 post-lesion, cylinder test on day 5 post-lesioning, (both tests also required a baseline assessment one day prior to lesioning) and amphetamine induced rotations on day 7 post-lesion. Full details of these tests are given earlier in section 3.3.2.5.

#### **5.3.1.6 Data analysis for behavioural tests**

Only behavioural data from animals showing correct cannula placement were included for data analysis presented in these studies. All behavioural analyses were assessed in a similar manner to those described in section 3.3.2.6. Briefly, in the adjusted stepping test, use of the contralateral and ipsilateral paw in both forehand and backhand directions post lesion were expressed as a % of pre-lesion use. Adjusted stepping scores between treatment groups were then compared using a 2-way ANOVA with Dunnett's *post-hoc* test at both 3 and 6 days post-lesion. Use of the contralateral and ipsilateral paw in the cylinder test, expressed as a % of total reaches, was also compared pre and post lesion between different treatment groups using a 2-way ANOVA with Bonferroni's *post-hoc* test. Finally, the number of amphetamine-induced rotations was plotted as both time course (5 min intervals) or

total rotations between 15-45 min and compared between treatment groups using a 2 or 1-way ANOVA respectively with a Bonferroni's *post-hoc* test. In all cases, data are expressed as mean  $\pm$  s.e.m. where  $P < 0.05$  was taken to be significant, with all statistical analysis performed using GraphPad Prism version 5.

#### **5.3.1.7 Immunohistochemical and HPLC protocols**

On the final day of dosing (day 8), animals were killed by CO<sub>2</sub> asphyxiation, the brains removed and segmented into rostral (striatum) and caudal (SN) segments. The striata from these animals were immediately dissected from the rostral segment and snap frozen on card-ice before storing at -80°C until further processing for HPLC measurement of dopamine levels. The SN segment on the other hand, was placed in 4% formalin for a period of at least 2 days to ensure fixation before processing for immunohistochemistry.

TH immunohistochemistry in the SNpc was performed exactly as described in section 3.3.2.7. Ionised calcium binding adaptor molecule 1 (IBA-1) and glial fibrillary acidic protein (GFAP) immunocytochemistry in the SNpc were performed exactly as described in section 4.3.1.7. Staining results in the SN were viewed on a Zeiss apotome microscope and recorded using Axiovision LE software (Carl Zeiss Ltd., UK).

Dopamine content of frozen striata was assessed using HPLC, exactly as described in section 3.3.2.9.

#### **5.3.1.8 Image and data analysis for immunohistochemistry and HPLC studies**

For TH immunohistochemistry in the SNpc, the average number of TH-positive cells in three adjacent sections for each animal across the three rostrocaudal levels (-4.8, -5.3, -5.8mm AP from bregma) were obtained as previously described in section 3.3.2.8, and the mean of these values taken per treatment group. Statistical comparisons were made between drug and vehicle-treated animals of mean TH-positive cell counts in the 6-OHDA lesioned side, expressed as % of the contralateral,

intact hemisphere, using a 1-way ANOVA with Dunnett's post-hoc test. For IBA-1 and GFAP staining in the SN, the average density or number of GFAP-positive cells between the contralateral and intact hemispheres in three adjacent sections at a central region of the SNpc (-5.3mm AP from bregma) were obtained, and the mean of these values taken per treatment group. Statistical comparisons of mean IBA-1 density measures or GFAP-positive cell counts in the 6-OHDA lesioned hemisphere, expressed as a % of the contralateral, intact hemisphere were made between drug and vehicle-treated animals using a 1-way ANOVA with Dunnett's post-hoc test. For HPLC analyses, dopamine content of striatal samples were converted from peak areas of the chromatogram using a calibration curve of pure reference standards and expressed as  $\text{ng.g}^{-1}$  protein. A 1-way ANOVA with Dunnett's *post-hoc* test was used to compare dopamine content of the 6-OHDA lesioned side, expressed as a % of the contralateral intact side between treatment groups. In all cases, data are expressed as mean  $\pm$  standard error of the mean (s.e.m.), where  $n$  represents the number of animals in each experimental group. Statistical analyses were performed using GraphPad Prism (version 5.0) and  $P < 0.05$  was taken to indicate significance.

#### **5.3.1.9 Materials and suppliers**

Details of all reagents, consumables and supplier contacts are included in Appendix I.

## 5.4 Results

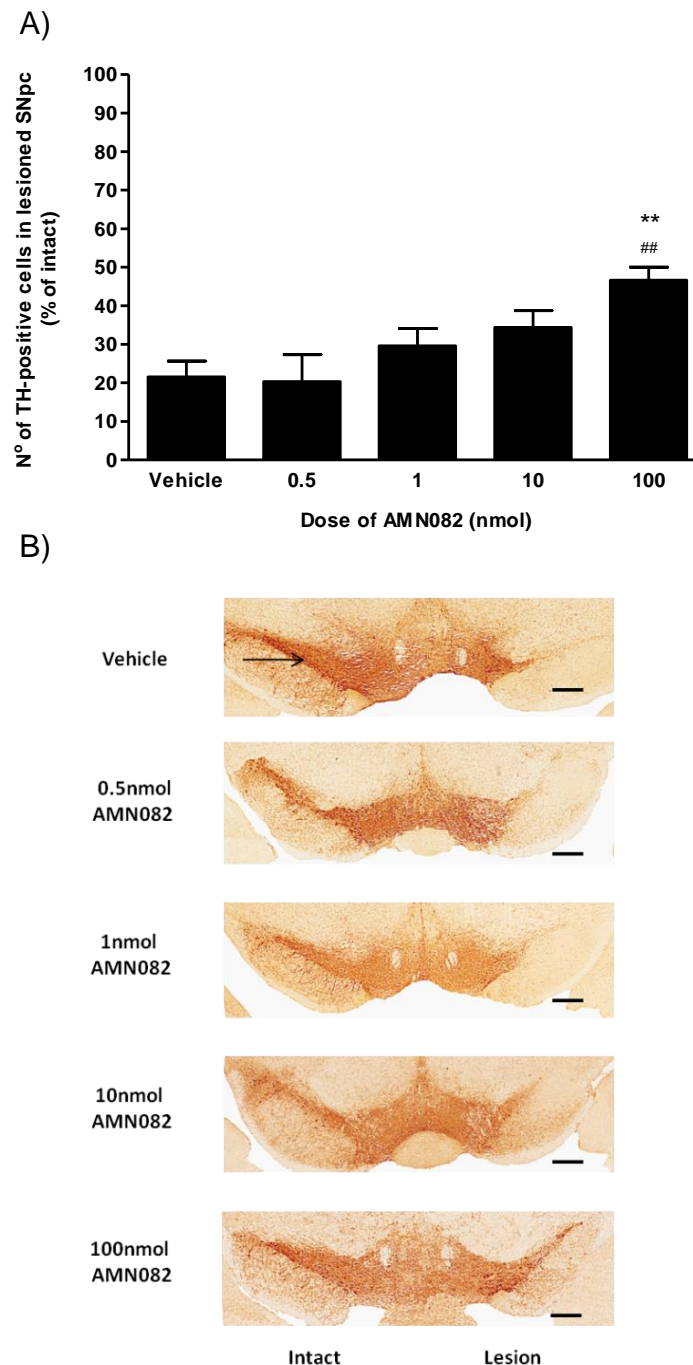
### 5.4.1 Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist, AMN082, in unilaterally 6-OHDA-lesioned rats

#### 5.4.1.1 Effects of sub-chronic AMN082 infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta

The number of TH-positive cells in nigral sections from animals treated supranigraly with sub-chronic AMN082 (0.5-100nmol) and those treated with vehicle (10% DMSO in PBS) are quantified in figure 5.1A. In the SNpc, the mean sum of TH-positive cells in triplicate sections obtained across three rostrocaudal levels declined from  $317.6 \pm 22.8$  in the intact hemisphere to  $66.0 \pm 15.1$  in the lesioned hemisphere of vehicle-treated 6-OHDA lesioned animals, (n=8). This equated to a deficit ~ 79% which was markedly smaller than previous studies highlighting the variability in lesion size in response to 6-OHDA. Supranigral treatment with AMN082 dose-dependently protected against TH-positive cell loss whereby maximal and significant protection was reached with a 100nmol dose of AMN082. The number of TH-positive cells remaining in the lesioned hemisphere following 100nmol AMN082 treatment (n=8), was  $150.3 \pm 9.3$  equivalent to  $46 \pm 3.4\%$  of the intact hemisphere, in contrast to  $21.5 \pm 4.2\%$  remaining in vehicle-treated animals ( $P < 0.01$ ; one-way ANOVA with Bonferroni's *post-hoc* test).

Representative nigral sections demonstrating immunoreactive TH-positive cells in the 6-OHDA lesioned animals treated with sub-chronic AMN082 (0.5-100nmol) are shown in figure 5.1B. The photomicrographs demonstrate supranigral infusion of AMN082 (100nmol) provides noticeable preservation of TH-positive cells in the lesioned hemisphere compared to vehicle-treated animals suggesting protection of dopaminergic neurones in these animals.

Figure 5.1 Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on tyrosine hydroxylase-positive cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.

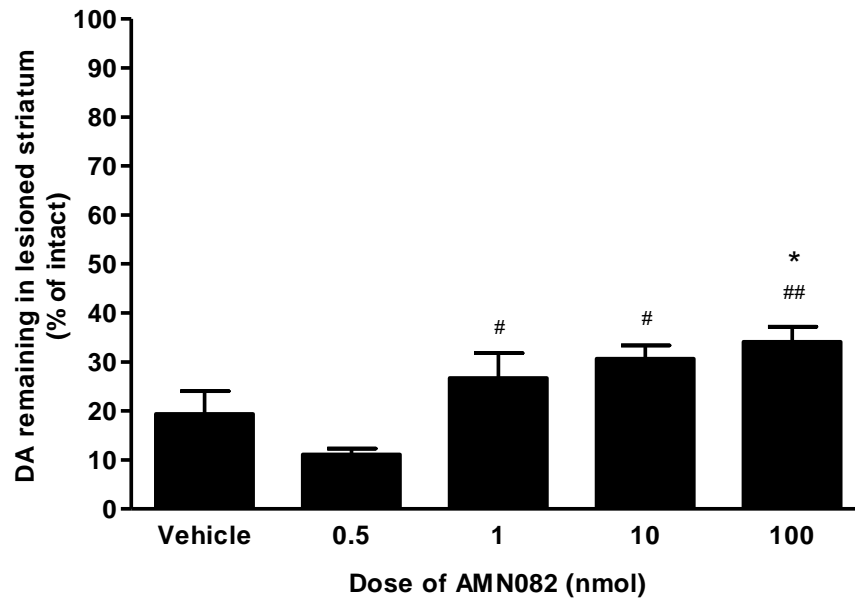


**Figure 5.1 (A)** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 10% DMSO in PBS) on the 6-OHDA induced loss of TH-positive cells in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082). \*\* (P < 0.01) indicates a significant difference compared to vehicle treated animals. ## (P<0.01) indicates significant difference compared to AMN082 (0.5nmol) treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). F = 4.986; df = 4,34. **(B)** Representative photomicrographs showing the levels of TH immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic AMN082 treatment. Arrow indicates SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **5.4.1.2 Effects of sub-chronic AMN082 infusion on dopamine content in the striatum**

Using HPLC, assessment of striatal dopamine content was shown to be preserved in the lesioned striatum of animals treated with AMN082. In vehicle-treated 6-OHDA lesioned animals, striatal dopamine content fell from  $18427 \pm 789 \text{ ng g}^{-1}$  in the intact hemisphere to  $3620 \pm 899 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $19.4 \pm 4.7\%$  ( $n=8$ ) of the intact hemisphere (figure 5.2). As with assessment of nigral TH, AMN082 treatment produced a dose-dependent preservation of striatal dopamine content, also reaching maximal effect with 100nmol AMN082 ( $P < 0.05$  versus vehicle treatment; 1-way ANOVA with Dunnett's *post-hoc* test), where dopamine content in the lesioned hemisphere remained at  $34.1 \pm 3.1\%$  of the intact hemisphere. At the lowest dose tested, AMN082 appeared to cause a decline in dopamine content, though this was not significant.

Figure 5.2: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist, AMN082, on dopamine content in the striatum of unilaterally 6-OHDA lesioned rats.



**Figure 5.2.** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 10% DMSO in PBS) on the 6-OHDA induced loss of dopamine content in the lesioned striatum expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082). \* (P<0.05) indicates a significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test). F = 6.950; df = 4,34. # (P<0.05) and ## (P<0.01) indicate significant difference compared to 0.5nmol AMN082 treated animals (1-way ANOVA with Bonferroni's *post-hoc* test).

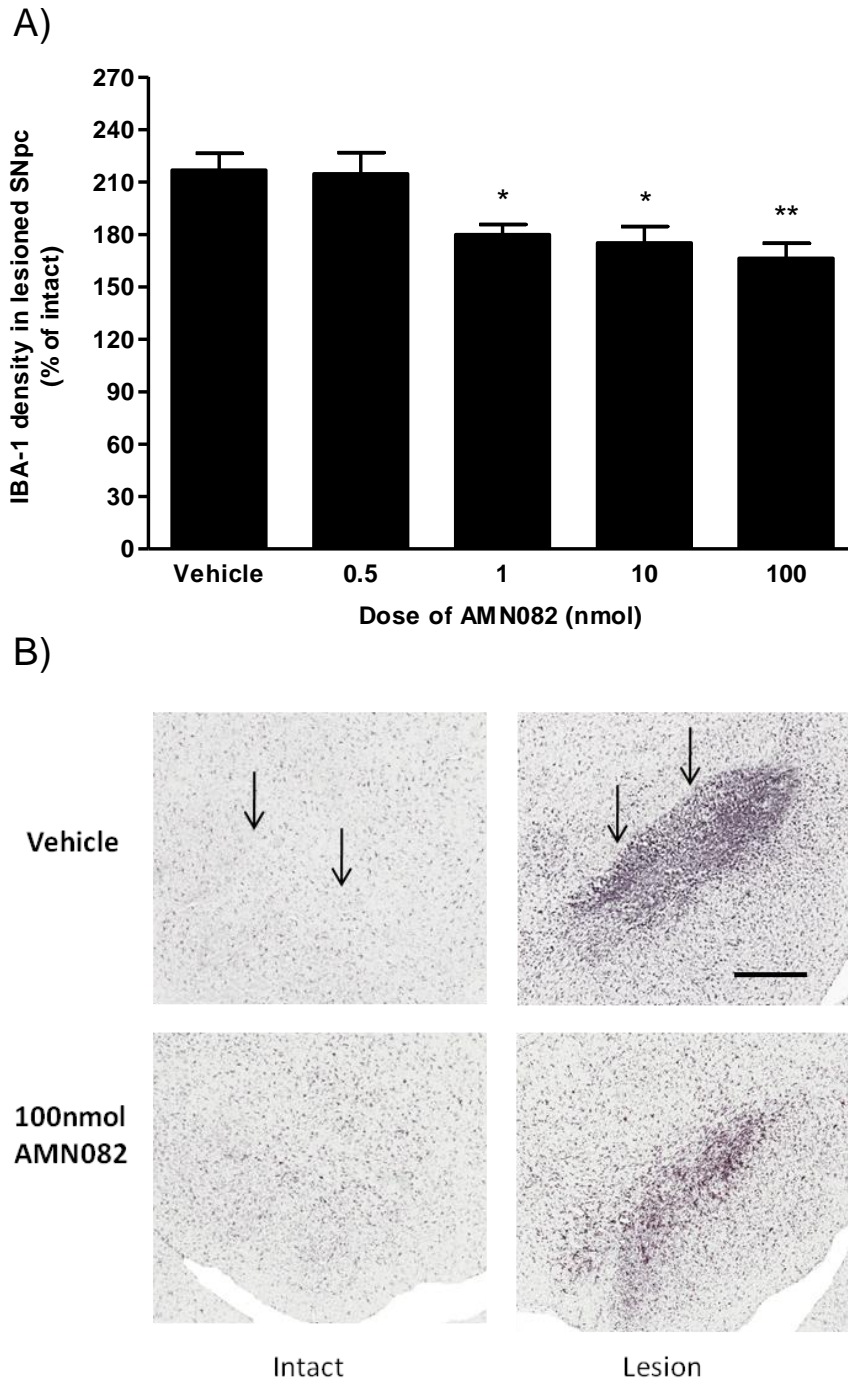
#### **5.4.1.3 Effects of sub-chronic AMN082 infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta**

As previously shown in Chapter 3, analysis of IBA-1, revealed a unilateral injection of 6-OHDA markedly increased microglial activation in the lesioned SNpc compared to the intact non-lesioned hemisphere (figure 5.3A). Following a unilateral 6-OHDA lesion, the mean total IBA-1 density at a central region of the SNpc increased to ~ 217% of the intact hemisphere in vehicle-treated animals (n=8). However, following treatment with AMN082, a graded decline in IBA-1 activation was observed with increasing doses of AMN082. Maximal effect was achieved with 100nmol AMN082 (n=8) whereby the increase in IBA-1 density was significantly reduced compared to vehicle treated animals, equating to a 166% increase in IBA-1 immunoreactivity with respect to the intact hemisphere ( $p<0.01$ ; one-way ANOVA with Bonferroni's *post-hoc* test).

Representative nigral sections showing IBA-1 immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic AMN082 (0.5-100nmol) are shown in figure 5.3B. Qualitative visual inspection revealed IBA-1-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in IBA-1-positive cells was observed, with a switch to ramified and amoeboid morphology. The photomicrographs demonstrate supranigral infusion of AMN082 (100nmol) clearly reduced levels of IBA-1 compared to the vehicle group in tune with the notion of a decreased inflammatory response in AMN082 treated animals.



Figure 5.3: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist, AMN082, on ionised calcium binding adaptor molecule 1 immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



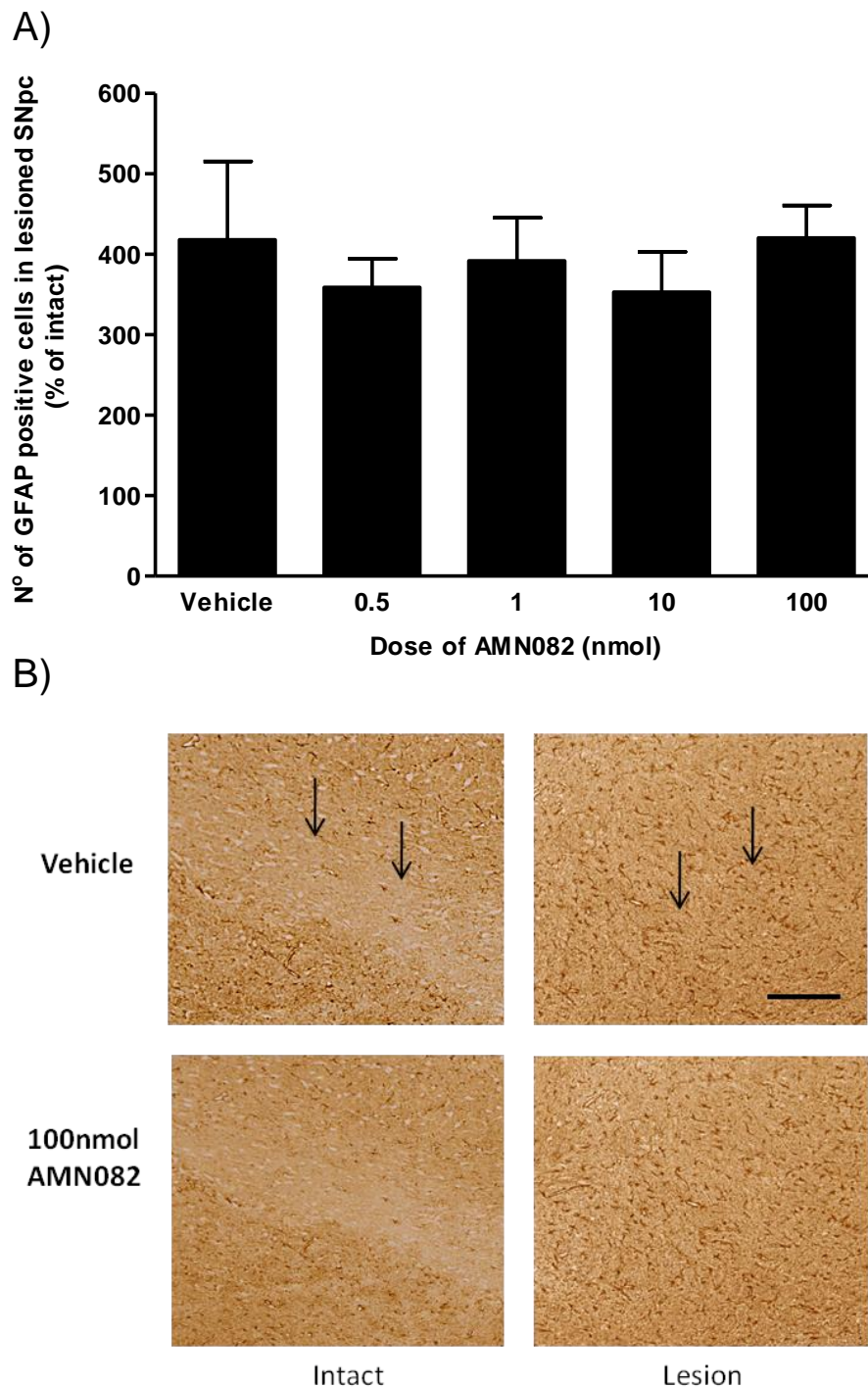
**Figure 5.3 (A)** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l of 10% DMSO in PBS) on the 6-OHDA induced elevation of IBA-1 density in the lesioned SNpc expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082). \* (P <0.05) and \*\* (P <0.01) indicate significant differences compared to vehicle treated animals (1-way ANOVA with Bonferroni's *post-hoc* test). F = 6.301; df = 4,34. **(B)** Representative photomicrographs demonstrating IBA-1 immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic AMN082 treatment. Arrows indicate boundary of SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **5.4.1.4 Effects of sub-chronic AMN082 infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta**

A marked increase in the astrocytic marker, GFAP, was also observed in the lesioned SNpc compared to the intact non-lesioned hemisphere in vehicle-treated animals (figure 5.4A). Following a unilateral 6-OHDA lesion, the mean total number of GFAP- positive cells increased to 418% of the intact hemisphere in vehicle-treated animals (n=8). Interestingly in comparison to findings with IBA-1, treatment with AMN082, at each dose tested, revealed no decline in GFAP immunoreactivity in the lesioned SNpc in comparison to vehicle-treated animals. Indeed, following AMN082 (100nmol) a 421% increase in GFAP-positive cells was observed with respect to the intact hemisphere attributing to a comparable level obtained in vehicle-treated animals.

Representative nigral sections demonstrating immunoreactivity to GFAP-positive cells in the 6-OHDA lesioned animals treated with sub-chronic AMN082 (0.5-100nmol) are shown in figure 5.4B. Qualitative analysis revealed GFAP-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in GFAP-positive cells displaying thickened star-shaped morphology was observed. The photomicrographs confirm findings noted above that animals supranigally infused with AMN082 (100nmol) demonstrated a comparable level of GFAP activation in the lesioned SNpc to that of vehicle-treated animals.

Figure 5.4: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on glial fibrillary acidic protein cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.



**Figure 5.4.** (A) The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 10% DMSO in PBS) on GFAP-positive cell number in the SNpc following a 6-OHDA lesion expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082).  $F = 0.2722$ ;  $df = 4,34$ . (B) Representative photomicrographs demonstrating GFAP immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic AMN082 treatment. Arrows indicate boundary of SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **5.4.1.5 Effects of sub-chronic AMN082 infusion on motor behaviour in 6-OHDA lesioned rats**

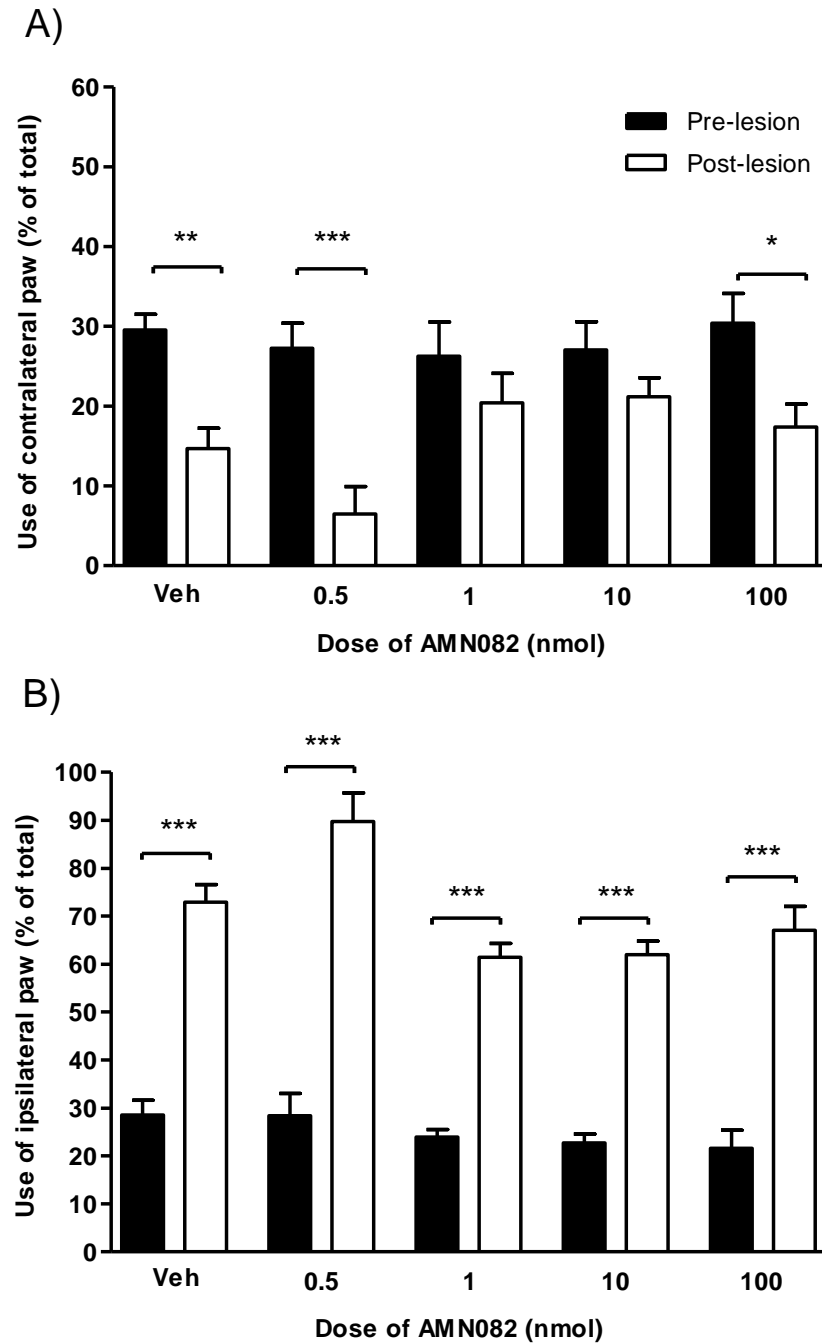
##### *Cylinder Test*

The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats were assessed using the cylinder test on day 5 post lesion.

As expected in vehicle-treated animals, there was a significant decrease in use of the contralateral paw from  $29.6 \pm 2.0\%$  pre-lesion to  $14.7 \pm 2.6\%$  post-lesion confirming that a unilateral 6-OHDA lesion induced a reduction of approximately 50% in contralateral paw use (figure 5.5A). Interestingly, 100 nmol AMN082, failed to preserve use of contralateral paw despite the observed preservation in post-mortem indices, demonstrating only a marginal improvement in post-lesion scores with respect to vehicle-treated animals. However, following treatment with both 1 and 10nmol AMN082 use of the contralateral paw was considerably preserved, failing to show a significant decline in use post-lesion. For example in 10nmol AMN082 treated animals, contralateral paw use equated to  $21.2 \pm 2.4\%$  of total paw use post-lesion in comparison to  $27.1 \pm 3.5\%$  pre-lesion equating to 78% preservation in contralateral paw use post-lesion following AMN082 treatment.

As previously described, a marked increase in ipsilateral paw use was observed in vehicle-treated animals following a 6-OHDA lesion in tune with the significant decrease in contralateral paw use. For example in vehicle-treated animals, ipsilateral paw use post lesion increased by ~73% with respect to pre-lesion score, compensating for an impaired contralateral paw. This effect was also apparent following treatment with AMN082, albeit to a lesser extent to that described in vehicle-treated animals (~62% increase in 10nmol AMN082 treatment group, figure 5.5B). Taken together, these findings suggest AMN082 can mediate some preservation of contralateral paw use following a 6-OHDA lesion, however the comparable increase in ipsilateral paw use post-lesion would suggest a degree of asymmetry remains in these treated animals.

Figure 5.5: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



**Figure 5.5.** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 10% DMSO in PBS) on contralateral (A) and ipsilateral (B) paw use expressed as a percentage of total paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion. For all, values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082) \* (P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) pre/post lesion factor, F = 35.59, df = 1,68 and for treatment factor, F = 1.805, df = 4,68. In (B) pre/post lesion factor, F = 354.4, df = 1,68 and for treatment factor, F = 7.043, df = 4, 68.

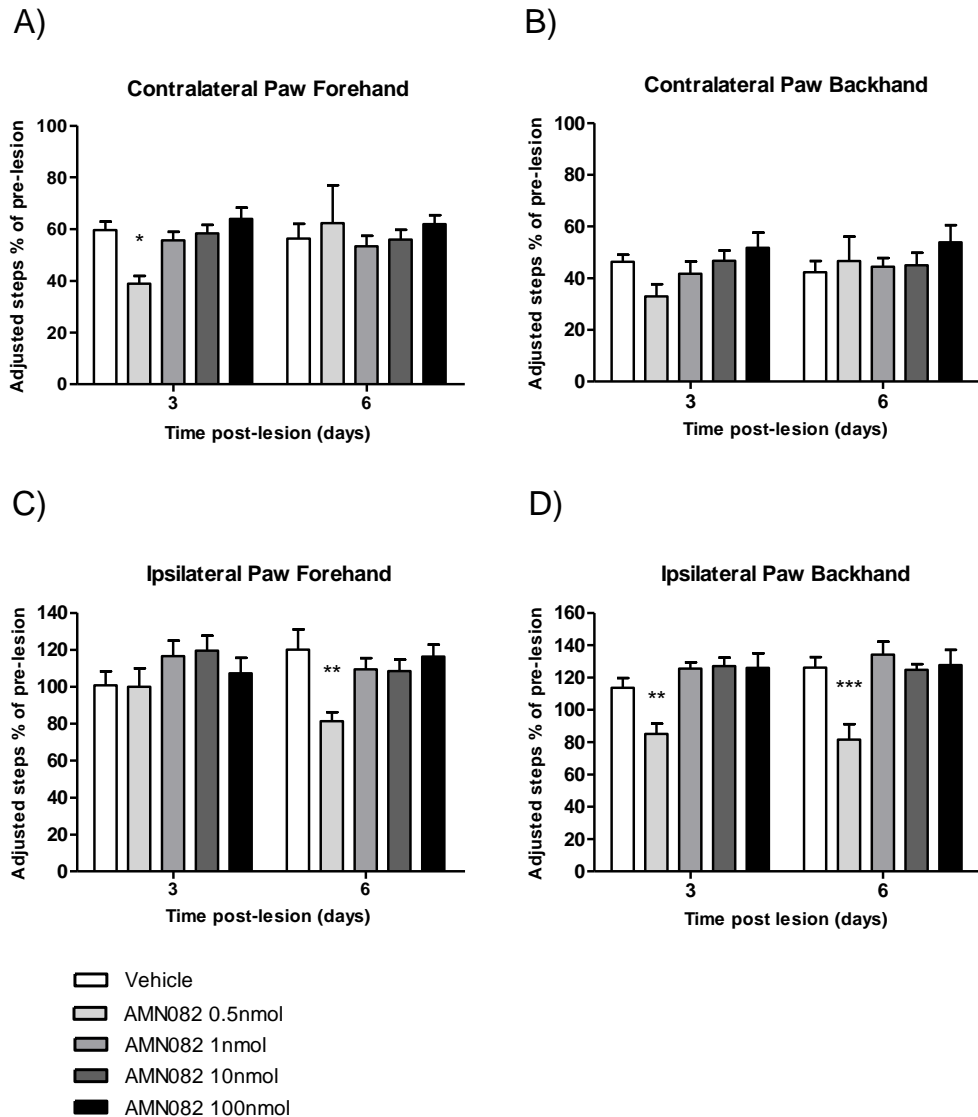
### *Adjusted Stepping*

The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 5.6. As predicted the contralateral paw demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $56.2 \pm 5.7\%$  at 6 days post-lesion in vehicle-treated animals, indicating unilateral 6-OHDA lesioning reduced the number of adjusted steps in this direction (figure 5.6A). Interestingly in contrast to the cylinder test, infusion of AMN082 failed to show any improvements in contralateral paw use with each of the doses tested.

In the backhand direction, there was also a marked reduction in contralateral paw use in vehicle-treated animals. This amounted to a decrease in adjusted steps from 100% pre-lesion to  $42.3 \pm 4.3\%$  at 6 days post-lesion confirming unilateral 6-OHDA-lesioning also reduced the number of adjusted steps in the backhand direction (figure 5.6B). However, similar to that observed in the forehand direction, no significant improvements in adjusted steps in any of the AMN082 treatment groups was observed.

As expected the ipsilateral paw (non-lesioned) in both the forehand and backhand direction showed no significant deficit in adjusted steps 3 or 6 days post-lesion in vehicle and AMN082 treated animals (figure 5.6C and D). Significant reductions in ipsilateral paw use were seen in AMN082 (0.5nmol) treated animals which may suggest animals in this treatment group were less responsive compared to vehicle-treated animals. Collectively these findings demonstrate supranigral sub-chronic AMN082 (0.5-100nmol) treatment does not significantly alter the number of adjusted steps in the contralateral paw, indicating that AMN082 does not improve motor asymmetry induced by unilateral 6-OHDA lesioning in this behavioural task.

Figure 5.6: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on motor function assessed using the adjusted stepping test in unilaterally 6-OHDA lesioned rats.



**Figure 5.6.** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l of 10% DMSO in PBS) on contralateral and ipsilateral paw use in the forehand and backhand direction (**A-D**) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion expressed as a percentage of pre-lesion score. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082). \*(P < 0.05), \*\* (P < 0.01) and \*\*\* (P < 0.001) indicates significant differences compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). In (**A**) for treatment factor, F = 1.150, df = 4, 68 and for days post lesion factor, F = 0.5041, df = 1, 68. In (**B**) treatment factor, F = 1.611, df = 4, 68 and for days post lesion, F = 0.5662, df = 1, 68. In (**C**) treatment factor, F = 3.110, df = 4, 68 and for days post lesion, F = 0.1078, df = 1, 68. In (**D**) treatment factor, F = 14.99, df = 4, 68 and for days post lesion, F = 0.5878, df = 1, 68.

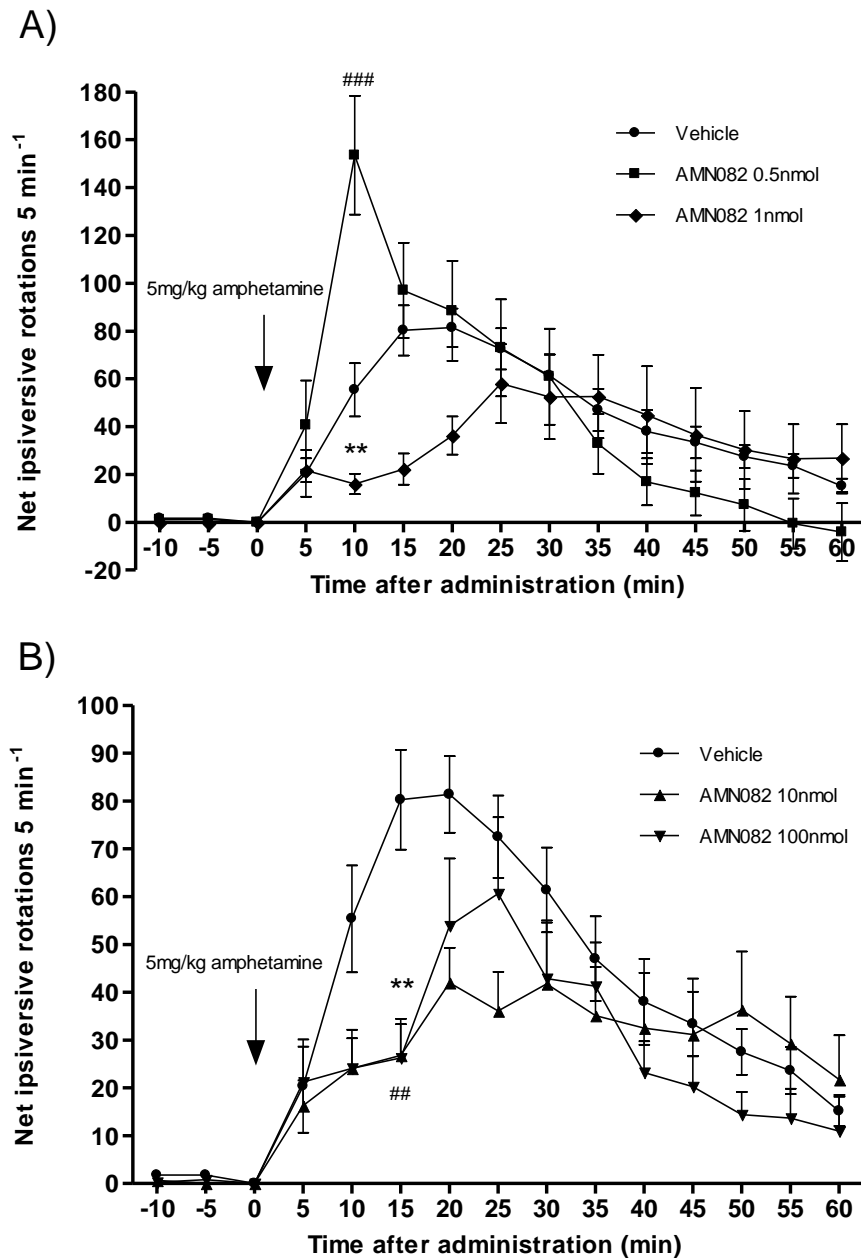
### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic AMN082 or vehicle are shown in figure 5.7. Both graphs A and B show the same vehicle group split with varying doses of AMN082 for clarity. Analysis of time-course in vehicle treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. Animals treated with 0.5nmol AMN082 demonstrated an unexpected initial increase in ipsiversive rotations (10 min post-injection,  $P < 0.001$ ) although no further significant effects with respect to vehicle-treated animals were reported. Following treatment with 1nmol AMN082, small decreases in ipsiversive rotations were observed, reaching significance at 10 min post injection of amphetamine ( $P < 0.01$ , 2-way ANOVA with Bonferroni's *post-hoc* test, figure 5.7A). Furthermore, figure 5.7B demonstrated 10 and 100nmol doses of AMN082 induced a moderate reduction in ipsiversive rotations particularly within the first 20 min following injection of amphetamine, with both doses reaching a significant reduction in rotations compared to vehicle-treated animals at 15 min post amphetamine ( $P < 0.01$ , 2-way ANOVA with Bonferroni's *post-hoc* test).

In figure 5.8, the mean total ipsiversive rotations were quantified over a 30 min period (15-45 min). The number of ipsiversive rotations by amphetamine ( $5\text{mg.kg}^{-1}$ ) in vehicle treated animals was  $414 \pm 46$  rotations in  $30\text{ min}^{-1}$ . AMN082 dose dependently decreased amphetamine-induced ipsiversive rotations reaching maximal effect with 100nmol AMN082. The total mean number of rotations equated to  $220 \pm 45$ , demonstrating a 47% reduction compared to vehicle treated animals, however this result was not significant ( $P > 0.05$ , 1-way ANOVA with Dunnett's *post-hoc* test). Nevertheless, this marked reduction suggests AMN082 treatment to an extent acts to reduce motor asymmetry induced by unilateral 6-OHDA lesioning.

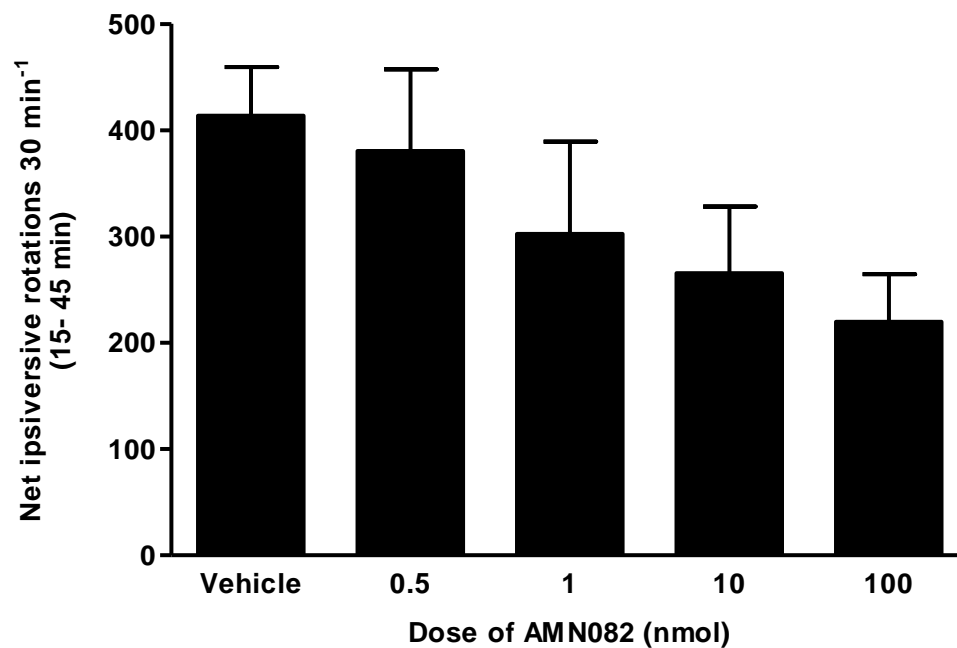


Figure 5.7: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 5.7.** The effects of sub-chronic supranigral infusion of AMN082 (0.5-100nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 10% DMSO in PBS) on amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. **(A)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and AMN082 (0.5, 1nmol) treated animals. **(B)** Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and AMN082 (10, 100nmol) treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082). **(A)** \*\* (P < 0.01; 0.5nmol AMN082) and ### (P < 0.001; 1nmol AMN082) indicates significant difference compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). **(B)** ## (P < 0.01; 10nmol AMN082) and \*\* (P < 0.01; 100nmol AMN082) indicates significant difference compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). For treatment factor F = 6.552; df = 4, 510 and for time F = 20.26; df = 14, 510.

Figure 5.8: Effects of sub-chronic supranigral infusion of the selective mGlu7 receptor allosteric agonist AMN082, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 5.8.** Total ipsiversive rotations induced by amphetamine in 30 min<sup>-1</sup> (15-45 min) in vehicle and AMN082 treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for vehicle, 0.5, 10 and 100nmol AMN082, n=7 for 1nmol AMN082).  $F = 1.589$ ;  $df = 4, 34$  (1-way ANOVA).

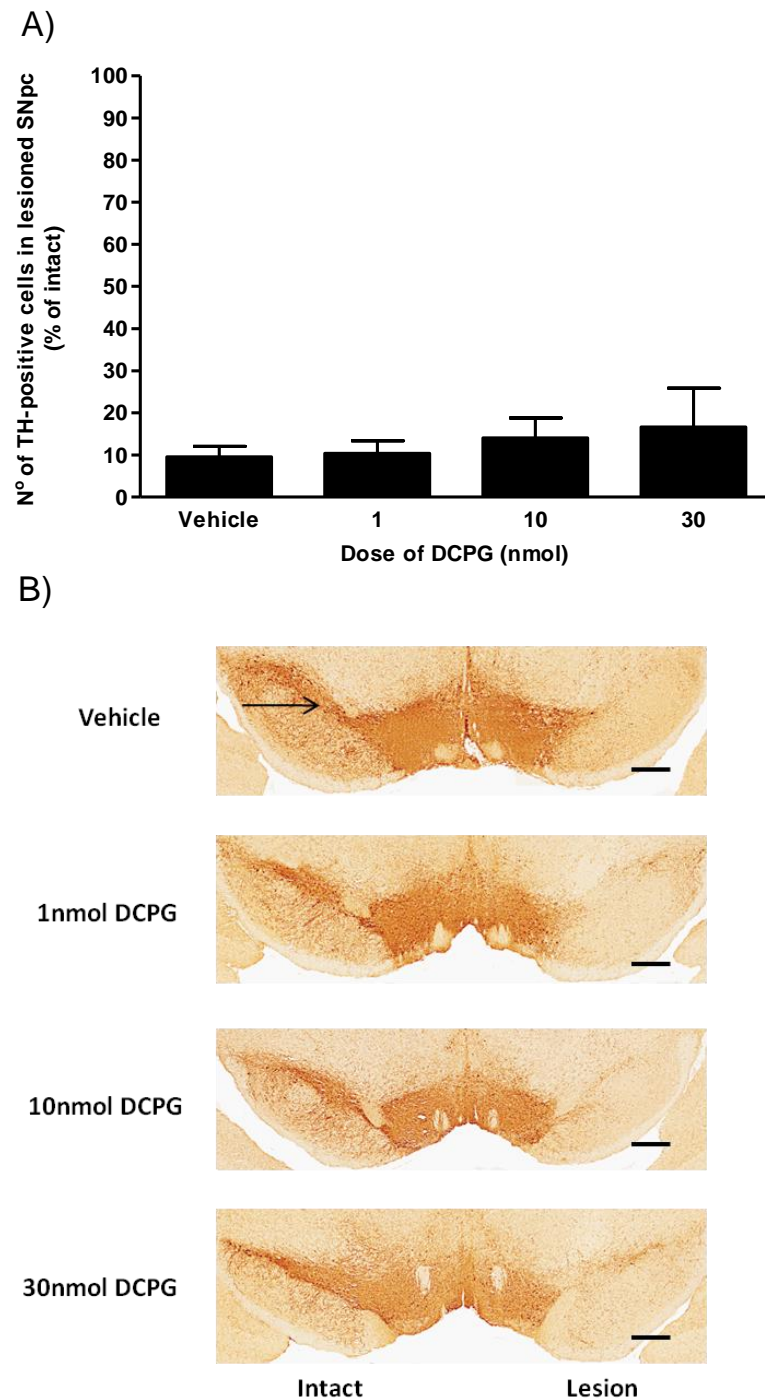
## **5.4.2 Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist, DCPG, in unilaterally 6-OHDA-lesioned rats**

### **5.4.2.1 Effects of sub-chronic DCPG infusion on tyrosine hydroxylase positive cells in the substantia nigra pars compacta**

In the SNpc, the mean sum of TH-positive cells in triplicate sections obtained across three rostrocaudal levels declined from  $257.6 \pm 17.2$  in the intact hemisphere to  $24.6 \pm 4.2$  in the lesioned hemisphere of vehicle-treated 6-OHDA lesioned animals ( $n=8$ ), demonstrating a full lesion with a deficit  $\sim 90\%$ . Supranigral treatment with DCPG (1-30nmol) failed to significantly protect TH-positive cells at each of the doses tested compared to vehicle treated animals. For example, the number of TH-positive cells remaining in the lesioned hemisphere following DCPG treatment (30nmol,  $n=8$ ), was  $40.4 \pm 7.1$  equivalent to  $16.6 \pm 9.3\%$  of the intact hemisphere in contrast to  $9.6 \pm 2.5\%$  remaining in the vehicle-treated animals (figure 5.9A).

Representative nigral sections demonstrating immunoreactive TH-positive cells in the 6-OHDA lesioned animals treated with sub-chronic DCPG (1-30nmol) are shown in figure 5.9B. The photomicrographs demonstrate following supranigral infusion of 30nmol DCPG a small number of TH-positive cells were present in the lesioned SNpc suggesting minor preservation in these treated animals. At the other doses tested, no noticeable preservation of TH-positive cells in the lesioned hemisphere was observed compared to vehicle-treated animals.

Figure 5.9: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on tyrosine hydroxylase-positive cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.

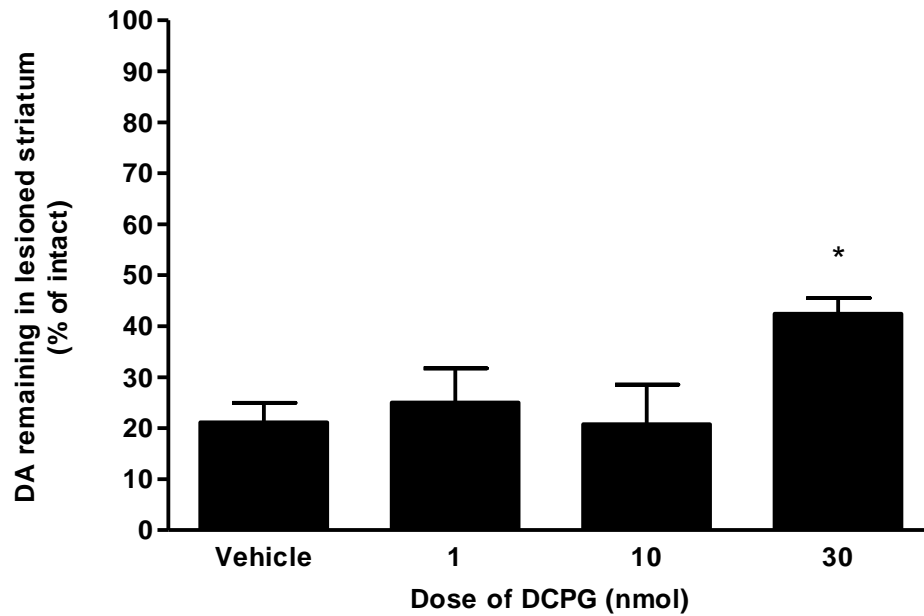


**Figure 5.9.** (A) The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 1x PBS) on the 6-OHDA induced loss of TH-positive cells in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups).  $F = 0.3554$ ;  $df = 3,28$ . (B) Representative photomicrographs showing the levels of TH immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic DCPG treatment. Arrow indicates SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **5.4.2.2 Effects of sub-chronic DCPG infusion on dopamine content in the striatum**

In vehicle-treated 6-OHDA lesioned animals, striatal dopamine content fell from  $16097 \pm 1099 \text{ ng g}^{-1}$  in the intact hemisphere to  $3524 \pm 748 \text{ ng g}^{-1}$  in the lesioned hemisphere, which represented a decline in dopamine to  $21.1 \pm 3.8\%$  ( $n=8$ ) of the intact hemisphere (figure 5.10). DCPG treatment at the lower doses tested (1-10nmol) failed to significantly preserve striatal dopamine content with respect to vehicle-treated animals. However, a more profound neuroprotective effect was observed with a high dose of DCPG (30nmol,  $P<0.05$  versus vehicle treatment; 1-way ANOVA with Dunnett's *post-hoc* test), where dopamine content in the lesioned hemisphere remained at  $42.4 \pm 3.1\%$  of the intact hemisphere.

Figure 5.10: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist, DCPG, on dopamine content in the striatum of unilaterally 6-OHDA lesioned rats.



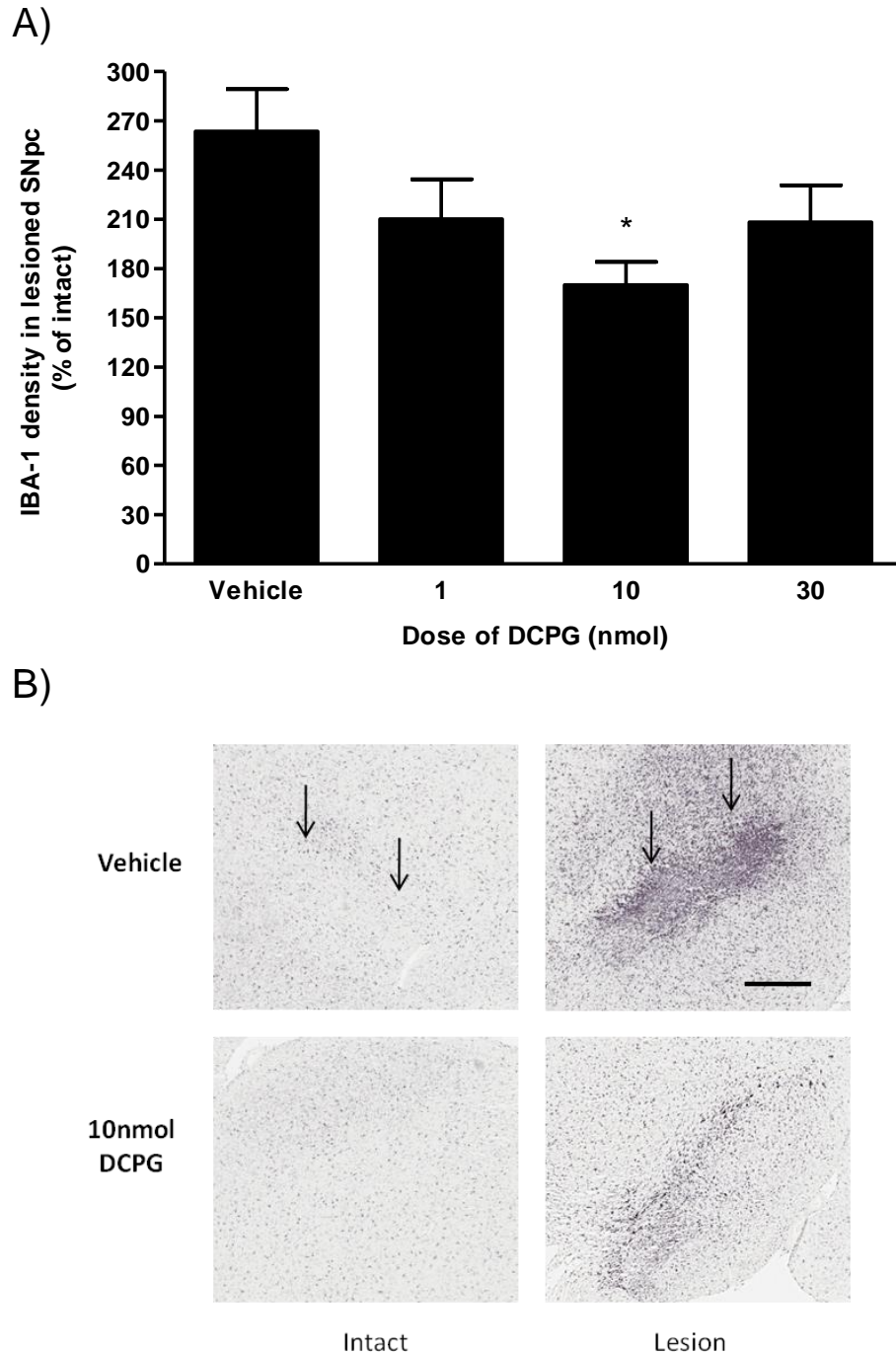
**Figure 5.10.** The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on the 6-OHDA induced loss of dopamine content in the striatum in the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups). \* (P<0.05) indicates a significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test) F = 3.238; df = 3,28.

#### **5.4.2.3 Effects of sub-chronic DCPG infusion on levels of calcium binding adaptor molecule 1 in the substantia nigra pars compacta**

Analysis of IBA-1, revealed a unilateral injection of 6-OHDA markedly increased microglial activation in the lesioned SNpc compared to the intact non-lesioned hemisphere (figure 5.11A). Following a unilateral 6-OHDA lesion, the mean total IBA-1 density at a central region of the SNpc increased to ~ 264% of the intact hemisphere in vehicle-treated animals (n=8). However, following DCPG treatment this increase in IBA-1 density was significantly reduced compared to vehicle-treated animals. Maximal reduction was achieved with a 10nmol dose of DCPG where only a 170% increase in IBA-1 immunoreactivity was observed with respect to the intact hemisphere ( $p<0.05$ ; one-way ANOVA with Dunnett's *post-hoc* test).

Representative nigral sections showing IBA-1 immunoreactivity in the 6-OHDA lesioned animals treated with sub-chronic DCPG (10nmol) or vehicle are shown in figure 5.11B. Qualitative analysis revealed IBA-1-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in IBA-1-positive cells was observed, with a switch to amoeboid morphology. The photomicrographs demonstrate supranigral infusion of DCPG (10nmol) markedly reduced levels of IBA-1 compared to vehicle-treated animals in tune with the notion of a decreased inflammatory response in these animals.

Figure 5.11: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist, DCPG, on ionised calcium binding adaptor molecule-1 immunoreactivity in striatal sections of unilaterally 6-OHDA lesioned rats.



**Figure 5.11.** (A) The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 1x PBS) on the 6-OHDA induced elevation of IBA-1-density in the SNpc on the lesioned side expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups). \* (P <0.05) indicates significant difference compared to vehicle treated animals (1-way ANOVA with Dunnett's *post-hoc* test) F = 3.042; df = 3,28. (B) Representative photomicrographs demonstrating IBA-1 immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic vehicle and DCPG (10nmol) treatment. Arrows indicate boundary of SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

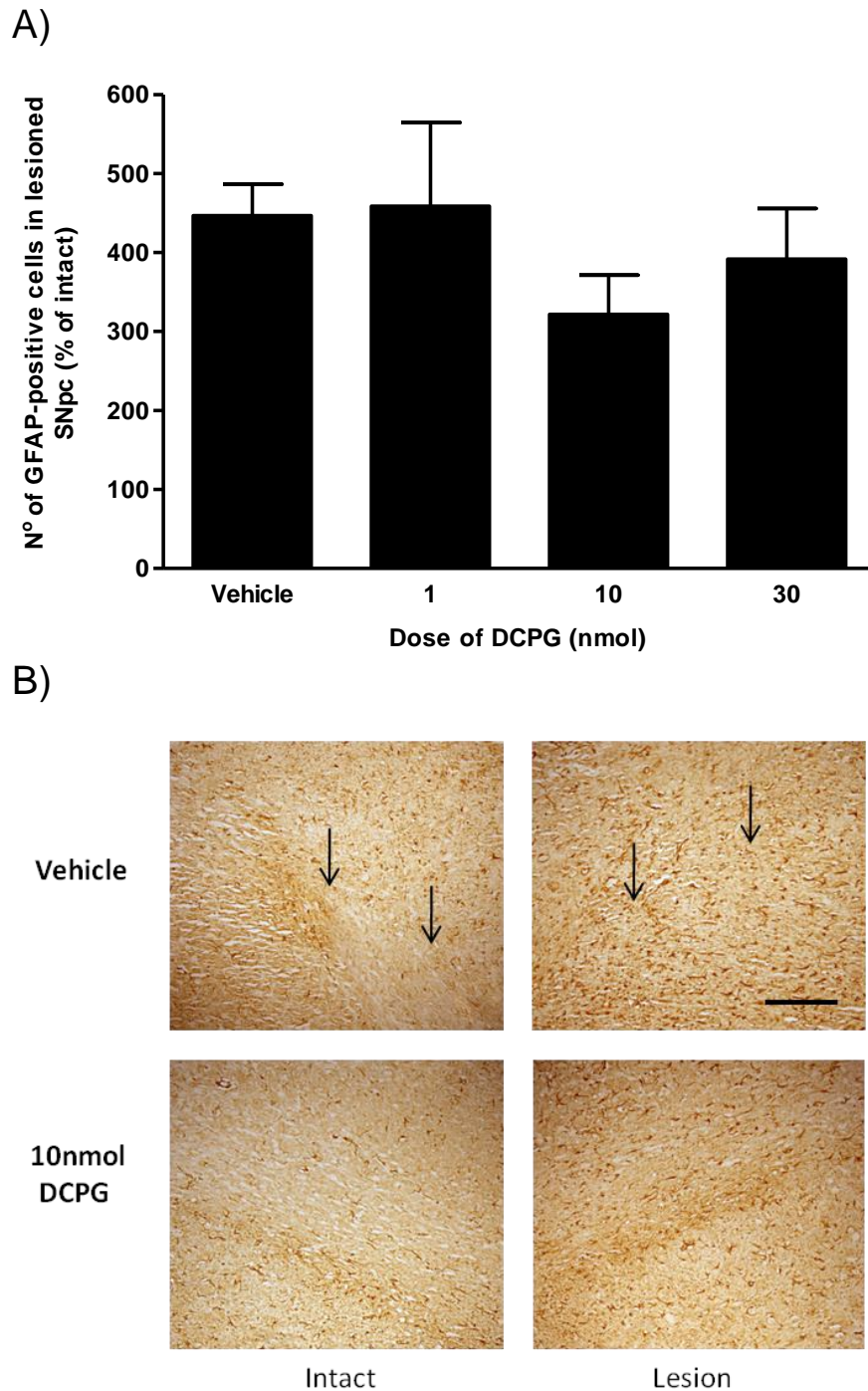


#### **5.4.2.4 Effects of sub-chronic DCPG infusion on levels of glial fibrillary acidic protein in the substantia nigra pars compacta**

A marked increase in the astrocytic marker GFAP was also observed in the lesioned SNpc compared to the intact non-lesioned hemisphere in vehicle-treated animals (figure 5.12A). Following a unilateral 6-OHDA lesion, the mean total number of GFAP- positive cells increased to 447% of the intact hemisphere in vehicle-treated animals (n=8). Following DCPG treatment (1-30nmol), no significant difference in GFAP immunoreactivity in the lesioned SNpc was observed on comparison to vehicle-treated animals. However, a marked decrease in GFAP-positive cells was seen following 10nmol DCPG equating to a 322% increase in GFAP-positive cell activation with respect to the intact hemisphere although this effect was not significant ( $p > 0.05$ ; one-way ANOVA with Dunnett's *post-hoc* test).

Representative nigral sections demonstrating immunoreactivity to GFAP-positive cells in 6-OHDA lesioned animals treated with sub-chronic DCPG (10nmol) or vehicle are shown in figure 5.12B. Qualitative analysis revealed GFAP-positive cells in the intact hemisphere displayed morphology typical of resting or quiescent cells. By contrast in the lesioned hemisphere a marked increase in GFAP-positive cells displaying thickened star-shaped morphology was observed. The photomicrographs demonstrate supranigral infusion of DCPG (10nmol) provided a clear reduction in GFAP activation compared to vehicle-treated animals consistent with the reduced levels of IBA-1 observed at this dose of DCPG.

Figure 5.12 Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on glial fibrillary acidic protein cell counts in nigral sections of unilaterally 6-OHDA lesioned rats.



**Figure 5.12.** (A) The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l PBS) on GFAP positive cell number in the SNpc following a 6-OHDA lesion expressed as a percentage of the intact side. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups)  $F = 0.8098$ ;  $df = 3,28$ . (B) Representative photomicrographs demonstrating GFAP immunoreactivity in nigral sections from 6-OHDA-lesioned animals following sub-chronic DCPG (10nmol) treatment or vehicle. Arrows indicate boundary of SNpc. Lesion side on the right; scale bar: 200 $\mu$ m.

#### **5.4.2.5 Effects of sub-chronic DCPG infusion on motor behaviour in 6-OHDA lesioned rats**

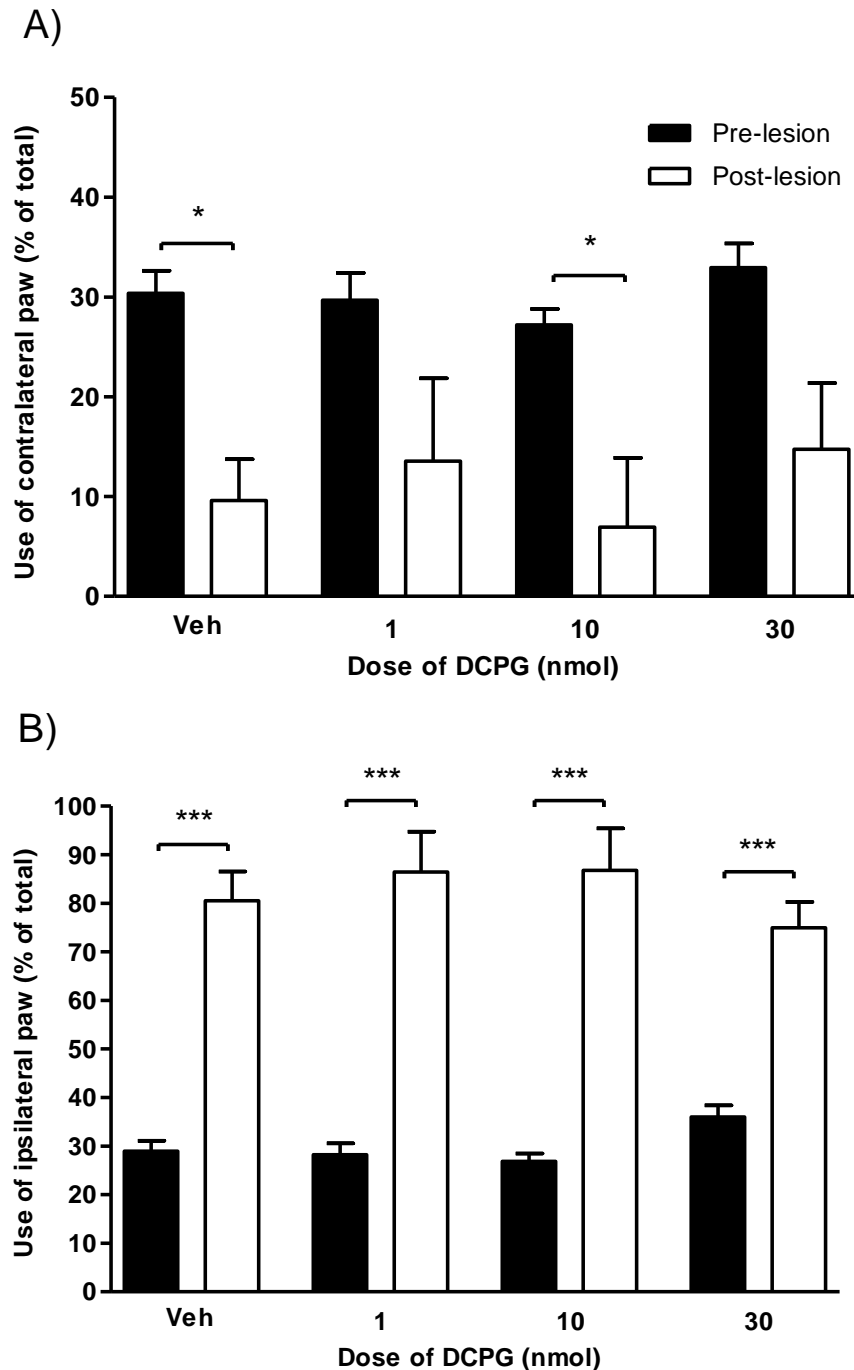
##### *Cylinder Test*

The effects of sub-chronic supranigral infusion of DCPG (1-30nmol) on spontaneous motor function following a unilateral 6-OHDA lesion of the SNpc in rats were assessed using the cylinder test on day 5 post lesion.

As expected in vehicle treated animals, there was a significant decrease in use of the contralateral paw from  $30.4 \pm 2.2\%$  pre-lesion to  $9.6 \pm 4.2\%$  post-lesion confirming that a unilateral 6-OHDA lesion induced a reduction of approximately 68% in contralateral paw use (figure 5.13A). In animals treated with DCPG (1-30nmol), assessment of post-lesion scores alone revealed use of contralateral paw was not significantly preserved at any of the doses tested, demonstrating only a marginal improvement with a high dose of DCPG (30nmol) compared to vehicle-treated animals. However 1 and 30nmol DCPG did not demonstrate a significant decline in contralateral paw use with respect to pre-lesion scores (2-way ANOVA with Bonferroni's *post-hoc* test) suggesting a degree of preservation in these animals.

As predicted, a marked increase in ipsilateral paw use was observed in vehicle treated animals following a 6-OHDA lesion in tune with the significant decrease in contralateral paw use. For example in vehicle treated animals, ipsilateral paw use post-lesion increased by ~81% with respect to pre-lesion score, compensating for an impaired contralateral paw. This effect was also apparent following treatment with DCPG in which a ~75% increase in ipsilateral paw use with respect to pre-lesion score was obtained (30nmol DCPG, figure 5.13B). These findings taken together with contralateral paw use confirms DCPG provides only a modest improvement in motor asymmetry induced by 6-OHDA lesioning in this behavioural task.

Figure 5.13: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on motor function assessed using the cylinder test in unilaterally 6-OHDA lesioned rats.



**Figure 5.13.** The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4  $\mu$ l) or vehicle (4 $\mu$ l PBS) on contralateral (A) and ipsilateral (B) paw use expressed as a percentage of total paw use assessed using the cylinder test at 5 days post a 6-OHDA lesion. For all, values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups) \* ( $P < 0.05$ ) and \*\*\* ( $P < 0.001$ ) indicates significant differences compared to pre-lesion score (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) pre/post lesion factor,  $F = 28.48$ ,  $df = 1,56$  and for treatment factor,  $F = 0.6486$ ,  $df = 3,56$ . In (B) pre/post lesion factor,  $F = 191.8$ ,  $df = 1,56$  and for treatment factor,  $F = 0.1011$ ,  $df = 3, 56$ .

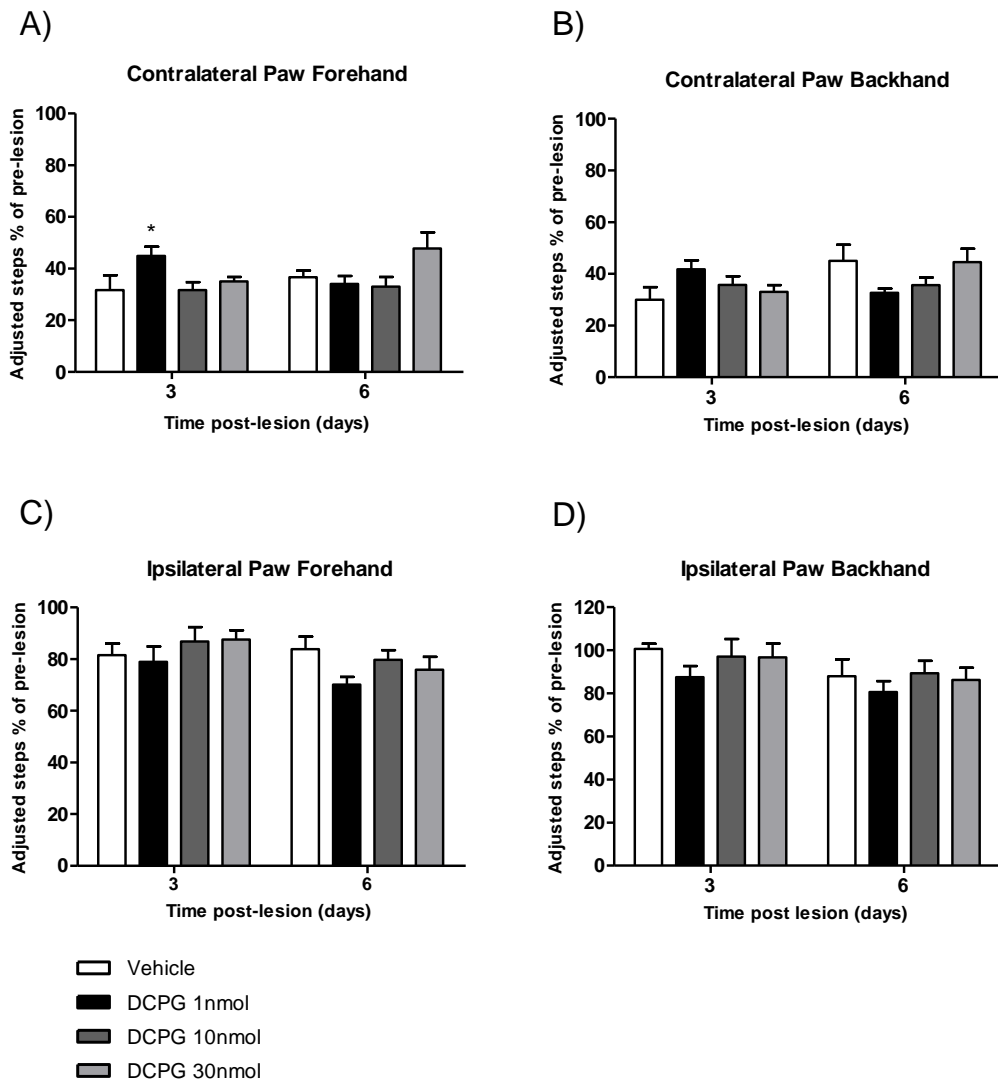
### *Adjusted Stepping*

The effects of sub-chronic supranigral infusion of DCPG (1-30nmol) on adjusted steps, 3 and 6 days following unilateral 6-OHDA lesioning in rats, are shown as a percentage of their pre-lesion scores in figure 5.14. As predicted the contralateral paw (lesioned) demonstrated a marked reduction in adjusted steps in the forehand direction, from 100% pre-lesion to  $36.7 \pm 2.6\%$  at 6 days post-lesion in the vehicle treated group, indicating unilateral 6-OHDA lesioning reduced the number of adjusted steps in this direction (figure 5.14A). Infusion of DCPG (1-10nmol) did not significantly preserve contralateral paw use at day 6 post-lesion. A small but significant preservation of contralateral paw use was observed at 3 days post-lesion following 1nmol DCPG ( $P < 0.05$  with respect to vehicle-treated animals), however this effect was lost at 6 days post-lesion. A degree of preservation of contralateral paw use was observed following 30nmol DCPG at 6 days post-lesion, equating to  $47.8 \pm 6.2\%$  of pre-lesion score, however this effect was not significant with respect to vehicle-treated animals.

In the backhand direction, there was also a marked reduction in contralateral paw use in vehicle-treated animals. This amounted to a decrease in adjusted steps from 100% pre-lesion to  $45.0 \pm 6.3\%$  at 6 days post-lesion confirming unilateral 6-OHDA-lesioning also reduced the number of adjusted steps in the backhand direction (figure 5.14B). Following DCPG treatment (1-30nmol) no significant preservation in use of the contralateral paw in the backhand direction was observed in any of the DCPG treated groups compared to vehicle-treated animals. As expected the ipsilateral paw (non-lesioned) in both the forehand and backhand direction showed no significant deficit in adjusted steps 3 or 6 days post-lesion in vehicle-treated animals (figure 5.14C and D).

Taken together, these findings demonstrate supranigral sub-chronic DCPG (1-30nmol) treatment does not significantly preserve contralateral paw use indicating DCPG, does not reduce the motor asymmetry induced by a unilateral 6-OHDA lesion in this behavioural task.

Figure 5.14: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on motor function assessed using the adjusted stepping test in unilaterally 6-OHDA lesioned rats.



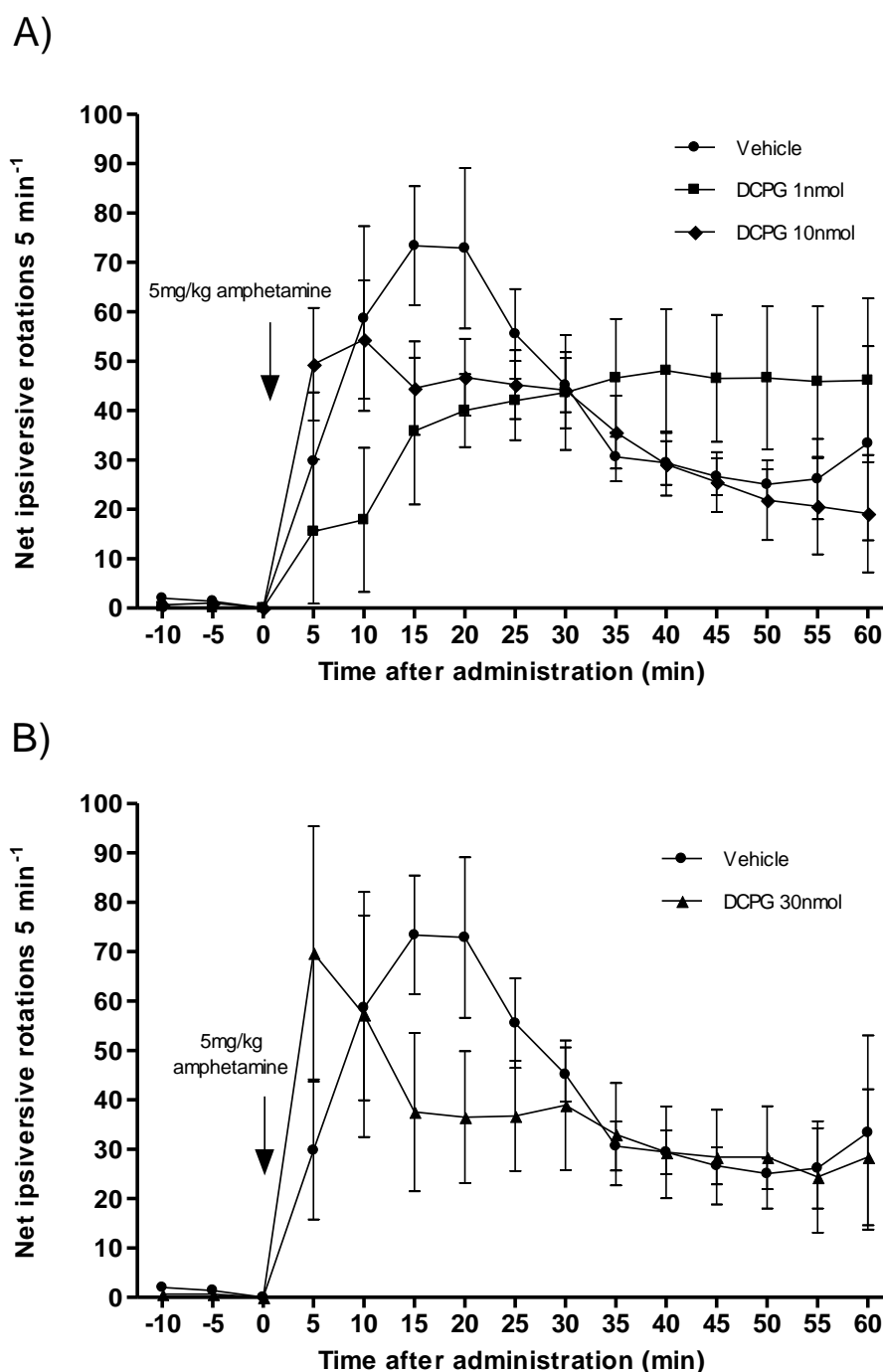
**Figure 5.14.** The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4μl) or vehicle (4μl PBS) on contralateral and ipsilateral paw use in the forehand and backhand direction (A-D) assessed using the adjusted steps test at 3 and 6 days post a 6-OHDA lesion expressed as a percentage of pre-lesion score. Values represent mean ± s.e.m. (n=8 for all treatment groups). \* (P < 0.05) indicates significant differences compared to vehicle treated animals (2-way ANOVA with Bonferroni's *post-hoc* test). In (A) for treatment factor, F = 2.327, df = 3,56 and for days post lesion factor, F = 0.5449, df = 1,56. In (B) treatment factor, F = 0.2001, df = 3,56 and for days post lesion, F = 2.285, df = 1,56. In (C) treatment factor, F = 1.579, df = 3, 56 and for days post lesion, F = 3.802, df = 1,56. In (D) treatment factor, F = 1.139, df = 3,56 and for days post lesion, F = 4.832, df = 1,56.

### *Amphetamine-induced rotations*

Amphetamine-induced ipsiversive rotations in unilaterally 6-OHDA lesioned rats treated with sub-chronic DCPG or vehicle are shown in figure 5.15. Both graphs A and B show the same vehicle group split with varying doses of DCPG for clarity. Analysis of time-course in vehicle-treated animals revealed amphetamine ( $5\text{mg.kg}^{-1}$ ) induced considerable ipsiversive rotations throughout a 60 min time period. However in animals treated with DCPG (1-10nmol) no significant difference between DCPG and vehicle-treated animals was observed from 0-60 min (figure 5.15A). A marked reduction in ipsiversive rotations between 15 and 20 min post injection of amphetamine was observed following 30nmol DCPG (figure 5.15B), however this effect was not significant when compared to vehicle-treated animals ( $P > 0.05$ ; 2-way ANOVA with Bonferroni's *post-hoc* test).

In figure 5.16, the mean total ipsiversive rotations were quantified over a 30 min period (15-45 min). The number of ipsiversive rotations by amphetamine ( $5\text{mg.kg}^{-1}$ ) in vehicle-treated animals was  $334 \pm 37$  rotations in  $30\text{ min}^{-1}$ . A dose dependent reduction in ipsiversive rotations was observed following treatment with DCPG treatment reaching maximal effect with 30nmol DCPG. At this dose the mean total number of rotations equated to  $240 \pm 64$ , demonstrating a 28% reduction compared to vehicle-treated animals, but again this result was not significant ( $P > 0.05$ , 1-way ANOVA with Dunnett's *post-hoc* test). This effect may reflect the small degree of protection observed in post-mortem indices following treatment with DCPG (30nmol).

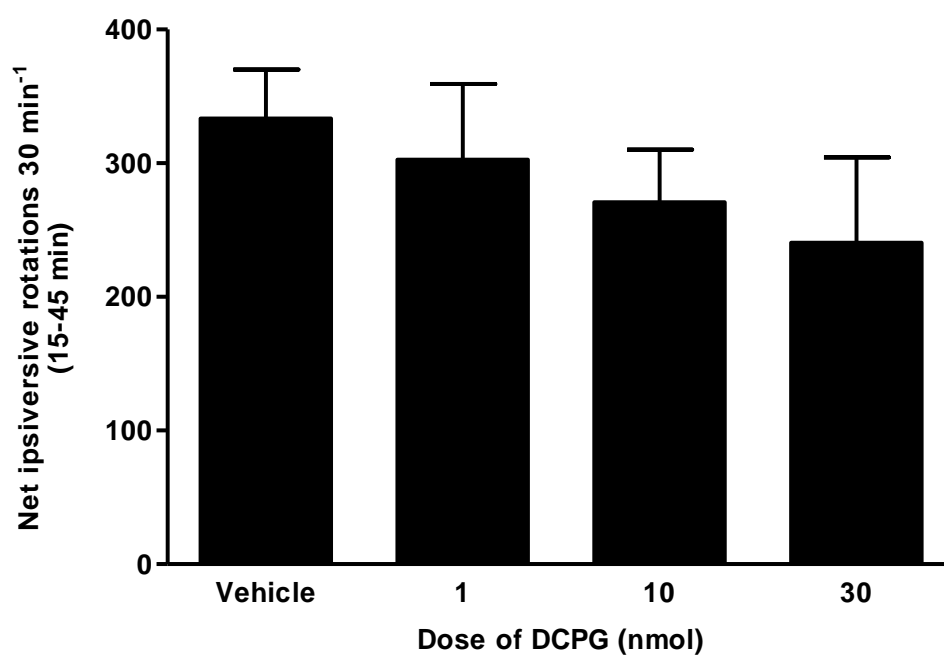
Figure 5.15: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 5.15.** The effects of sub-chronic supranigral infusion of DCPG (1-30nmol in 4 $\mu$ l) or vehicle (4 $\mu$ l 1x PBS) on amphetamine induced rotations in unilaterally 6-OHDA lesioned rats. (A) Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and DCPG (1, 10nmol) treated animals. (B) Time course of ipsiversive rotations induced by amphetamine (5mg.kg<sup>-1</sup>) in vehicle and DCPG (30nmol) treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups). For treatment factor F = 0.5506; df = 3,420 and for time F = 9.636; df = 14, 420.



Figure 5.16: Effects of sub-chronic supranigral infusion of the selective mGlu8 receptor agonist DCPG, on motor function assessed following amphetamine-induced rotations in unilaterally 6-OHDA lesioned rats.



**Figure 5.16.** Total ipsiversive rotations induced by amphetamine in 30 min<sup>-1</sup> (15-45 min) in vehicle and DCPG treated animals. Values represent mean  $\pm$  s.e.m. (n=8 for all treatment groups).  $F = 0.6341$ ;  $df = 3, 28$  (1-way ANOVA).

## 5.4 Discussion

In this chapter we demonstrate for the first time, using the selective allosteric agonist AMN082, that mGlu7 receptor activation mediates neuroprotection and preservation of motor function in the 6-OHDA rat model of PD. In addition, the effects of AMN082 were complemented by a reduction in an inflammatory marker, suggesting a reduced inflammatory response may contribute in part to this protective effect. In contrast, activation of mGlu8 receptors using the selective agonist DCPG was less promising revealing modest protection in post-mortem indices with little to no preservation in motor function. DCPG did, however, reduce indices of microglial activation, suggesting it too may confer some degree of anti-inflammatory action.

To investigate the potential of targeting mGlu7 receptors to mediate functional neuroprotection, the selective allosteric agonist AMN082 was tested in 6-OHDA-lesioned rats. AMN082 administered 1h before and for 7 days post a 6-OHDA lesion significantly protected the nigrostriatal tract. This was shown by the preservation of TH-positive cells in the SNpc and the reduction in 6-OHDA-induced loss of striatal dopamine content, whereby maximal effect was achieved with a 100nmol dose of AMN082. In addition these studies revealed that the degree of neuroprotection was sufficient to preserve certain motor functions or symmetries that would otherwise be lost following a unilateral injection of 6-OHDA. Thus, marked preservation of contralateral paw use was shown in the cylinder test following AMN082 treatment in addition to a reduction in amphetamine-induced ipsiversive rotations. However, not all behavioural tests gave a positive outcome; in the adjusted steps test AMN082 did not combat the loss of contralateral paw stepping induced by 6-OHDA. Furthermore, there is some degree of mismatch between the effective doses of AMN082. While significant preservation of TH-positive cells in the SNpc was only seen at the highest dose of 100nmol, striatal dopamine content was significantly preserved at all three doses examined. Similarly, efficacy in the cylinder test was noted with 1 and 10nmol doses, but not the 100nmol dose which did, conversely provide a significant reduction in amphetamine-induced rotations. The reasons behind this mismatch are not clear, although the behavioural outcomes generally are well allied with striatal dopamine suggesting preservation of dopamine in the terminal regions of the nigrostriatal tract

is a more essential requirement for improved motor function than protection at the level of the nigra. Furthermore it is not surprising greater preservation was observed in the striatum since the toxin was introduced directly into the cell bodies so it may be more difficult to protect at the nigral level compared to the terminals. Indeed, more robust protection is often seen in the 6-OHDA lesioned rats at the striatal level, reported using AMPA potentiators (Murray *et al.*, 2003) and nicotinic agonists (Visanji *et al.*, 2006). Nonetheless, overall, these studies are clearly indicative of a protective effect afforded by this mGlu7 allosteric agonist in the 6-OHDA rat model of PD.

The neuroprotective effects of AMN082 are certainly consistent with the distribution studies described in Chapter 2 which suggested mGlu7 may be ideally situated to mediate preservation of the nigrostriatal system demonstrating high intensity staining in the dorsal tier of the SNpc as well as in the striatum. Indeed as with mGlu4, mGlu7 has been found on excitatory (presumed glutamatergic) terminals in the SNpr (Kosinski *et al.*, 1999) which receives excitatory inputs from the STN but also the pedunculopontine nucleus and frontal cortex (Carter, 1982; Di Loreto *et al.*, 1992; Kita & Kitai, 1987). However the presence of mGlu7 mRNA in the STN (Kosinski *et al.*, 1999; Messenger *et al.*, 2002) strongly supports their expression on terminals of STN efferents which co-excite both segments of the substantia nigra (SNpr and SNpc) via the subthalamonigral pathway (Valenti *et al.*, 2005). Although the majority of mGlu7 receptors are assumed pre-synaptic in origin, low levels of mGlu7 immunoreactivity have been found at post-synaptic dendritic sites in both the GP (Bradley *et al.*, 1999; Kosinski *et al.*, 1999) and striatum (Kosinski *et al.*, 1999). Electrophysiological studies to date however, have found no evidence for a post-synaptic action of group III mGlu receptors in the GP (Valenti *et al.*, 2003) although such an action in the striatum remains to be determined. Therefore at this moment in time, it is most likely that the receptors in the SNpc are similarly pre-synaptic receptors.

Having demonstrated a neuroprotective effect of AMN082, this raises the question as to what potential mechanisms may underlie this effect. Since this is the first study of its kind to demonstrate a neuroprotective role for mGlu7 receptors, there is little

published evidence to guide us in this respect. However on the basis of previous work undertaken in this laboratory, the likely mechanisms include inhibition of glutamate release in the SNpc to facilitate protection from glutamate-mediated excitotoxicity. Previous studies in our lab have shown that mGlu7 receptors, using AMN082, reduces glutamate release from nigral tissue *in vitro*. When given alongside a sub-threshold concentration of L-AP4, AMN082 produced a concentration-dependent inhibition of release (Broadstock *et al.*, 2011). Interestingly, when added to SNpr tissue prisms alone, AMN082 (0.01 – 100  $\mu$ M which extended beyond its EC<sub>50</sub> of 0.064 $\mu$ M) failed to modify [3H]-D-aspartate release. Furthermore in a separate study, AMN082 was shown to potentiate L-AP4-mediated inhibition of EPSCs in the rat basolateral amygdala (Ugolini *et al.*, 2008) suggesting a potentiating rather than direct agonist for this agent. However electrophysiological studies confirming these effects in the SNpc are awaited to clarify this notion.

Identification of mGlu7 receptors on primary cultures of rat astrocytes, suggests additional non-neuronal mechanisms may also have been involved in mediating neuroprotection with AMN082 (Yao *et al.*, 2005). As mentioned in the previous chapter, it is thought that following insult with a pathological stimulus such as 6-OHDA, substantial increases in pro-inflammatory cytokines and cell adhesion molecules occur, whereupon activated microglia are recruited to the site of injury and cluster around dopaminergic neurones to be phagocytosed (Banati *et al.*, 1998; Bronstein *et al.*, 1995). In astrocyte cultures from mGlu4 knock-out mice, L-AP4 (300  $\mu$ M) at a concentration known to occupy mGlu7 receptors was shown to reduce the production of a pro-inflammatory chemokine, **Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES)** (Besong *et al.*, 2002). This suggests that activation of glial mGlu7 receptors may well contribute to the regulation of such pro-inflammatory chemokines in mediating anti-inflammatory actions. Interestingly treatment of AMN082 at all the doses tested did not demonstrate any significant effect on GFAP activation, yet a significant reduction in activation of microglia in the SNpc was observed supporting the notion of an anti-inflammatory mechanism. However the lack of effect on GFAP activation would favour the glutamate hypothesis as the predominant mechanism here, in which AMN082 facilitates protection from glutamate-mediated excitotoxicity in the SNpc.

An important observation from this study was that the lesion size in vehicle-treated animals was approximately 80% which is considerably smaller than the desired ~90% seen in previous chapters, suggesting a degree of experimental variability in these animals. Whilst it is unlikely a reduced lesion size would affect the ability of AMN082 to mediate protection, the reduced behavioural impairment as a result of a more partial lesion in vehicle-treated animals may account for the less pronounced effect of AMN082 with respect to preservation of motor function. Indeed at the immunohistochemical level, AMN082 (100nmol) demonstrated a comparable level of protection to that seen with VU0155041 (100nmol, ~45% of intact hemisphere), yet in behavioural indices the effects of VU0155041 were far more pronounced. Another point worthy of mention when considering the level of protection seen with AMN082, is the fact that it was administered supranigrally in 10% DMSO, which is known to be neurotoxic. Indeed, in the study of Battaglia et al., (2006) 50% DMSO in saline (the vehicle for PHCCC) given i.p. led to an increase in MPTP toxicity. However our lab has shown no increase in toxicity in the vehicle (10% DMSO in PBS) compared to animals treated only with PBS, assessed by loss of TH in the lesioned striatum, suggesting this is unlikely to influence the degree of preservation seen here with AMN082 (Austin., 2007, PhD Thesis).

It would have been highly desirable to have confirmed the receptor selectivity of AMN082 at mGlu7 in a follow-up study using a selective mGlu7 antagonist. However the only currently available compound MMPiP whilst effective *in vitro* has not yet provided compelling antagonism of mGlu7 receptors *in vivo*. This has been exemplified in a recent study where MMPiP was found to block only limited actions of mGlu7 signalling and failed to block mGlu7-mediated inhibition of EPSPs across the Schaffer collateral CA1 synapse (Niswender *et al.*, 2010). Given this poor profile, the use of MMPiP *in vivo* cannot yet be justified as interpretation of these results would still be ambiguous. Thus despite the promising neuroprotective effects revealed with AMN082, confirmation of the involvement of the mGlu7 receptor in these responses remains to be confirmed. This confirmation is essential for interpretation of the effects of AMN082, given recent preliminary reports that AMN082 is rapidly metabolised *in vivo*, with its metabolite displaying binding affinity for a wide range of targets including DAT, NAT and SERT (Sukoff-Rizzo *et*

*al.*, 2010). The direct injection into the SNpc in this study most likely circumvents many of these issues with metabolite formation, favouring an mGlu7 mediated effect with AMN082 using this experimental paradigm. However since completion of these experiments, a recent report has suggested a pharmacologically rich profile for AMN082. In this study off target effects were assessed by measuring inhibition of binding using a screening concentration of 10 $\mu$ M AMN082 at 71 GPCR, transporter, ion channel and enzyme targets revealing significant activity at 26 targets including DAT (92%), NAT (87%),  $\mu$  opioid peptide receptor (MOP, 91%) and acetylcholinesterase (100%) with potencies likely to exceed that at mGlu7 (Brodbeck *et al.*, 2010). Thus owing to the rich pharmacology of AMN082 we cannot be certain the neuroprotective effects seen here are mGlu7 mediated until a subtype selective antagonist is used or mGlu7 knockout studies are undertaken. Furthermore, these findings highlight the need to develop more selective compounds to assess fully the potential of targeting mGlu7 receptors.

The final objective of these studies was to investigate the potential of targeting mGlu8 receptors to mediate functional neuroprotection, using the selective agonist DCPG in the 6-OHDA rat model of PD. The studies described here demonstrate that following administration of DCPG 1h before and for 7 days post a 6-OHDA lesion failed to demonstrate significant protection of the nigrostriatal tract. This was shown by a lack of preservation of TH-positive cells in the SNpc and no significant preservation of motor function in these animals. Interestingly, assessment of striatal dopamine revealed significant preservation following a high dose of DCPG (30nmol). Indeed this greater level of protection afforded at the striatal level was also observed in studies with AMN082, again demonstrating preservation at the nigral level is more difficult to achieve when 6-OHDA is introduced directly into the cell bodies. However what remains unclear here is why protection afforded at the striatal level failed to provide any significant preservation of motor function in behavioural outcomes, although there was a trend to reduce amphetamine-induced rotations at this dose. This may suggest the dopamine present in the lesioned striatum may not be of neuronal origin whereby microdialysis experiments have revealed intraneuronal pools of dopamine that exist following a 6-OHDA lesion that are insensitive to evoked release (Zetterstrom *et al.*, 1988). Indeed further studies would be required to

ascertain the physiological state of the dopamine detected here. For example, a microdialysis study would be of worth to examine dopamine efflux following stimulation of potassium to confirm neuronal derivation. An additional suggestion is that DCPG may alter TH activity thereby increasing dopamine content or act to reduce dopamine turnover, although both these possibilities remain speculative at this point.

The lack of neuroprotection seen following treatment with DCPG is perhaps not surprising since our immunohistochemical studies investigating the distribution of group III mGlu receptors (discussed in Chapter 2), revealed generally low intensity staining for mGlu8 in the basal ganglia particularly within the SNpc. Furthermore previous studies in our lab have reported a lack of efficacy of DCPG to inhibit glutamate release from nigral slices (Broadstock *et al.*, 2011). DCPG, failed to inhibit release over a wide concentration range (0.03 – 10  $\mu$ M, spanning the estimated EC<sub>50</sub> of 31 nM) when applied either alone or in combination with L-AP4 (Broadstock *et al.*, 2011). Interestingly in a parallel study, injection of DCPG into the SNpr did produce a dose-dependent reversal of akinesia, however, this effect was not inhibited by pre-treatment with CPPG, suggesting it was driven through an off-target action of DCPG, independent of group III mGlu receptor activation (Broadstock *et al.*, 2011). Although the identity of such a target is yet to be identified, an additional low affinity non-mGlu, non iGlu receptor target was described for DCPG when it was first characterised (Thomas *et al.*, 2001) raising a concern over the selectivity for DCPG at mGlu8.

Electrophysiological studies employing whole-cell patch clamp recordings from dopaminergic neurones of the rat SNpc, confirmed DCPG had no effect on evoked EPSPs in the SNpc (Valenti *et al.*, 2005). Application of 300 nM (S)-3,4-DCPG, a concentration 10-fold higher than the EC<sub>50</sub> of this compound at recombinant mGluR8, produced no effect on the excitatory transmission in the SNpc suggesting that mGluR8 does not play a role in modulating transmission on the subthalamonigral pathway. Furthermore Lopez *et al.*, (2007) found a similar lack of efficacy of DCPG against haloperidol-induced catalepsy following intranigral administration, and to reverse 6-OHDA-induced akinesia following intrapallidal injection (Beurrier *et al.*,

2009) demonstrating a lack of target potential of mGlu8 receptors to provide symptomatic relief in PD.

While a protective role is ruled out, DCPG at a single dose (10nmol) did show a significant anti-inflammatory response by decreasing levels of IBA-1. This effect is important as it indicates that the protection seen with VU0155041 (in the previous chapter) and above with AMN082 are *not* likely to be solely dependent on reducing levels of IBA-1, otherwise DCPG would have also shown clear protection here. Whilst this does not rule out an inflammatory mechanism these findings certainly suggest this is not the predominant mechanism in mediating neuroprotection which may ultimately be driven through an inhibition of glutamate release. Of course it is possible neuroprotection implicates multiple mechanisms and an anti-inflammatory mechanism may still provide an integral contribution to achieve this desired outcome. Taken together, these findings suggest that mGlu8 demonstrates the least promise as a potential novel group III mGlu target for the treatment of PD, shown here by its failure to provide functional neuroprotection in the 6-OHDA rat model of PD. However, it is unclear how the limited pharmacology of DCPG has contributed to this supposition. Only when further studies are performed using more selective mGlu8 agents over a wider dose range confirmed with corresponding antagonist or exclusive mGlu8 receptor knockout studies, can a neuroprotective role for mGlu8 be definitively ruled out.

To conclude, the studies presented in this chapter demonstrate selective activation of mGlu7 receptors using the allosteric agonist AMN082 can mediate neuroprotection and preservation of motor function in the 6-OHDA rat model of PD. However a degree of uncertainty remains over the potential of targeting mGlu7 receptors owing to the recent unearthing of a pharmacologically rich profile for AMN082 and further studies are clearly required to address this caveat. As for mGlu8, these studies along with findings in the literature reveal little evidence to pursue targeting this group III mGlu receptor subtype for disease modification in PD. However it is unclear at this stage how the potential of targeting mGlu8 has also been hindered by a lack of selective pharmacological ligands. Collectively, the data described here would imply



that the mGlu7 group III mGlu receptor subtype, rather than mGlu8 is the more likely target for providing functional neuroprotection in PD.

## **Chapter 6: General conclusions**

Current treatments for PD to date have relied heavily on reinstating dopaminergic transmission either with L-DOPA or dopamine agonists. Whilst this has proven to be extremely effective at treating the symptoms of the disease, this approach does little to combat the progressive degeneration. This failure affects patients' long-term health because the increasing doses of drug required to stabilize worsening symptoms often result in disabling adverse effects, such as L-DOPA induced dyskinesia and psychosis (Stocchi *et al.*, 1997). Clearly then the development and use of neuroprotective agents for PD represents one of the most important goals for researchers in this field. Indeed, the need for such therapies is now stronger than ever since as average human life expectancy increases so will the prevalence of PD, and therefore suffering and economic burden caused by loss of earning and cost of care will too rise. Thus there is a huge medical and economic incentive to find an effective treatment for this disorder.

In PD, loss of striatal dopamine evokes numerous downstream changes within the basal ganglia, including increased firing of the STN (Vila *et al.*, 1999). The subsequent increased release of glutamate in the STN target areas such as the SNpr and GPi, leads to inhibition of thalamocortical feedback which contributes to the generation of motor deficits associated with PD. Importantly, the STN also sends direct projections to the SNpc (Iribe *et al.*, 1999; Smith & Bolam, 1990), which under parkinsonian conditions may lead to a parallel increase in glutamate release and contribute to the progressive degeneration in the SNpc via an excitotoxic mechanism (Rodriguez *et al.*, 1998). Accordingly, reducing STN firing via subthalamotomy or deep brain stimulation reduces symptoms in animal models and PD patients (Alvarez *et al.*, 2005; Peppe *et al.*, 2004; Windels *et al.*, 2005) while chemical inactivation of the STN (through kainic acid lesioning), also reduces nigrostriatal tract damage induced by the dopaminergic toxin, 6-hydroxydopamine (6-OHDA) (Piallat *et al.*, 1996). Given these surgical procedures are invasive, expensive and available in few centres world-wide, alternative strategies for combating the effects of increased STN activity are desirable. One such approach may be the pharmacological inhibition of glutamate release from STN terminals in the SN and group III mGlu receptors are one of a number of possible targets that might achieve this and thereby sequester degenerative processes in PD.

The  $G_i / G_o$  – coupled group III mGlu receptors, which in the brain comprise mGlu4,7 and 8 are found on pre-synaptic elements of glutamatergic synapses where they serve a modulatory role (Conn & Pin, 1997). Of particular relevance here, previous *in vitro* electrophysiological studies have shown that activation of group III mGlu receptors using the broad spectrum agonist, L-AP4 inhibits glutamate transmission across the subthalamonigral synapse (Wittmann *et al.*, 2001). This finding has since been supported by work from our laboratory showing that activation of group III mGlu receptors in the SN leads to a reduction of glutamate release both *in vitro* and, more importantly, *in vivo* (Austin *et al.*, 2010). Armed with this knowledge, the studies in this thesis were undertaken to assess whether activation of group III mGlu receptors in the SNpc, could offer benefit in PD by protecting dopaminergic neurones from 6-OHDA induced toxicity.

In Chapter 2, a preliminary study was conducted to assess group III mGlu receptor distribution in the basal ganglia via immunohistochemistry. These studies paid particular attention to mGlu8, where expression studies were limited. Previous studies have been conducted to assess mGlu4 and mGlu7 receptor distribution and our observations were in good agreement with these findings revealing particularly intense staining for both receptor subtypes in the SNpc, suggesting these receptors may be ideally positioned for pharmacological manipulation. Furthermore, these studies revealed the presence of mGlu8 throughout the basal ganglia. Generally, the levels of mGlu8 expression seen here appeared to be considerably lower than that seen for both mGlu4 and 7 in each of the basal ganglia nuclei with the exception of the striatum, although these were not quantifiably compared. These findings therefore revealed that all three group III mGlu receptor subtypes are potential pharmacological targets for future investigation.

The neuroprotective potential of targeting group III mGlu receptors has already been suggested from findings of Vernon *et al.*, (2005), who demonstrated the ability of sub-chronic L-AP4, administered intranigally, to preserve dopaminergic cell bodies and terminals of the nigrostriatal tract in 6-OHDA-lesioned rats. This study however, did not investigate whether the protection of dopaminergic neurones resulted in preservation of motor behaviour. Therefore the initial studies in Chapter 3 of this

thesis, aimed to confirm the neuroprotective ability of L-AP4 in 6-OHDA-lesioned rats, and additionally investigate whether this protection translated into preservation of motor behaviour.

The studies detailed in Chapter 3 confirmed sub-chronic L-AP4, administered supranigrally 1 hour before and for 7 days post 6-OHDA-lesioning afforded neuroprotection shown by preservation of TH, AADC immunohistochemistry and neurochemical assessment of striatal dopamine content. Importantly, this study also revealed that the protection observed against a 6-OHDA lesion translated to preservation of motor function. This was apparent by a significant preservation of contralateral paw use in the adjusted steps test and cylinder test of forelimb akinesia following L-AP4 treatment, as well as a reduction in the degree of amphetamine-induced rotations. These positive behavioural outcomes indicated that the improvement in striatal dopamine levels in the lesioned hemisphere reduced striatal dopamine asymmetry between this and the intact hemisphere sufficiently to improve motor function. Furthermore, in a follow-up study, neuroprotection mediated by L-AP4 was shown to be significantly inhibited with prior administration of a selective group III mGlu receptor antagonist CPPG in both immunohistochemical and neurochemical indices. The ability of L-AP4 to improve motor function was also significantly compromised following pre-treatment with CPPG in all three behavioural measures. The findings confirmed the protective effects mediated by L-AP4 were driven by a group III mGlu receptor dependent mechanism.

In Chapter 4, studies commenced into identifying which of the known group III mGlu receptor subtypes was responsible for mediating this protective effect seen with L-AP4, firstly by examining mGlu4 receptors. Administration of the novel mGlu4 selective PAM VU0155041 was found to significantly protect the nigrostriatal tract. This was evidenced by a preservation of TH-positive cells in the SNpc and reduction in 6-OHDA-induced loss of striatal TH and DDC immunoreactivity. Furthermore, these preserved markers of nigrostriatal tract integrity were reflected in elevated dopamine content in the striatum of VU0155041 versus vehicle-treated animals. Of important functional significance, VU0155041 also preserved motor function post 6-OHDA lesion. Significant preservation of contralateral forelimb use was shown in

both the cylinder and adjusted stepping test following VU0155041 treatment in addition to a reduction in amphetamine-induced ipsiversive rotations, reflecting reduced asymmetry between the lesion and intact hemispheres in this hemiparkinsonian model. Pre-treatment with CPPG prior to VU0155041, markedly attenuated both the behavioural end-points and the post-mortem indices of preserved nigrostriatal tract function, confirming VU0155041 produced these effects via a receptor mediated process.

Numerous mechanisms may underlie this mGlu4 receptor-mediated neuroprotection, however following intranigral administration, as undertaken in this study, the two most likely mechanisms include inhibition of glutamate release in the SNpc and protection thereby from glutamate-mediated excitotoxicity, or reduced inflammatory actions. Support for the glutamate hypothesis was already available in the form of electrophysiological studies which had revealed that STN-evoked EPSCs in dopaminergic neurones of the SNpc were inhibited by the mGlu4 PAM, PHCCC (Valenti *et al.*, 2005). In addition, studies conducted in our laboratory have recently shown that PHCCC inhibits depolarisation-evoked release of [<sup>3</sup>H]-D-aspartate from slices of rat SN *in-vitro* (Broadstock *et al.*, 2011). *In vivo* microdialysis studies using VU0155041 would have helped strengthen this *in vitro* support. Although time did not permit undertaking microdialysis during the course of this thesis, such studies are currently underway and should help shed further light on whether inhibition of glutamate release in the SNpc contributes to the neuroprotective effects seen following mGlu4 receptor potentiation.

The studies of Chapter 4 have however, provided new evidence in support of an additional, anti-inflammatory contribution potentially mediated by astroglial mGlu4 receptors. The marked decrease in GFAP-positive cells observed here following VU0155041 treatment may suggest a role for mGlu4 receptor activation in restricting 6-OHDA-induced astroglial-driven neurotoxicity *in vivo*. Treatment with the mGlu4 PAM also led to a significant reduction in activation of microglia in the SNpc of 6-OHDA rats, as shown by the reduced levels of IBA-1 in the lesioned hemisphere of VU0155041-treated animals. Therefore, although these mechanisms have only just begun to be explored, it is tempting to suggest a component derived from activation

of astroglial mGlu4 receptors may contribute to the overall protective potential of targeting these receptors in PD. However, these data should be interpreted with an element of caution, as decreases in microglial markers may reflect a secondary effect to neuroprotection mediated by VU0155041 through alternative mechanisms. Furthermore, in the preliminary fluorescent immunohistochemical studies undertaken at the close of Chapter 4, there was little evidence for co-localisation of mGlu4 receptors with GFAP. Therefore any anti-inflammatory component is certainly more likely to reflect an indirect effect via stimulation of neuronal mGlu4 receptors, rather than a direct effect of stimulating mGlu4 receptors localised on the glial cells themselves. Owing to this uncertainty, further studies are no doubt required to probe the potential of an mGlu4-derived anti-inflammatory action in mediating neuroprotection in rodent models of PD.

Interestingly the level of protection achieved following mGlu4 receptor activation alone was below that achieved with L-AP4. This effect may be due to not reaching the optimal dose of VU0155041 and higher doses should be considered in future studies. However, this finding would also suggest additional group III mGlu receptor subtypes i.e. mGlu7 and mGlu8 may too provide neuroprotective effects. Indeed, the studies presented in Chapter 5 reveal the mGlu7 allosteric agonist, AMN082, administered 1h before and for 7 days post a 6-OHDA lesion significantly protects the nigrostriatal tract. This was shown by a preservation of TH-positive cells in the SNpc and reduction in 6-OHDA-induced loss of striatal dopamine. Furthermore, this study revealed neuroprotection following treatment with AMN082 also led to a degree of preservation of motor function post-lesion. This was shown by a marked preservation of contralateral paw use in the cylinder test following AMN082 treatment, in addition to a reduction in amphetamine-induced ipsiversive rotations.

In an attempt to investigate a potential mGlu7-mediated anti-inflammatory mechanism, treatment with AMN082 resulted in a significant decline in IBA-1 activation however no effect on GFAP activation was observed. This would imply an astrocytic mGlu7 neuroinflammatory mechanism is unlikely to be mediating the neuroprotection observed here and again may reflect an indirect effect via stimulation of neuronal mGlu4 receptors. The most likely mechanism for mGlu7-mediated

neuroprotection is via inhibition of glutamate release in the SNpc to facilitate protection from glutamate-mediated excitotoxicity. From studies in our laboratory, AMN082 was shown to inhibit depolarisation-evoked [3H]-D-aspartate from slices of rat SN *in vitro* (Broadstock *et al.*, 2011). Additionally, AMN082 has been shown to potentiate L-AP4-mediated inhibition of EPSCs in the rat basolateral amygdala (Ugolini *et al.*, 2008) providing further weight to the glutamate hypothesis.

Following treatment with the selective mGlu8 agonist DCPG, whilst displaying preservation of striatal dopamine, demonstrated no immunohistochemical protection at the level of the SN or preservation of motor behaviour. This overall lack of protection seen with DCPG was not too surprising since our immunohistochemical studies investigating the distribution of group III mGlu receptors in Chapter 2, revealed generally low intensity staining for mGlu8 in the basal ganglia particularly within the SNpc. Furthermore, previous studies in our lab have reported a lack of efficacy of DCPG to inhibit glutamate release from nigral slices (Broadstock *et al.*, 2011). An important observation in this study was that whilst DCPG did not protect at the level of the SNpc, significant reductions in IBA-1 activation were observed. This mismatch between an anti-inflammatory action and lack of protection would suggest an astrocytic-mediated neuroinflammatory response may in fact be insufficient on its own to provide neuroprotection or serves more of a secondary role here. Thus, in the absence of any inhibition of glutamate release, i.e. following mGlu8 receptor activation (Broadstock *et al.*, 2011), this anti-inflammatory action alone is insufficient to provide significant protection while when, as in the case of mGlu4 and mGlu7, this effect occurs most likely in concert with inhibition of glutamate release (Broadstock *et al.*, 2011) to offer protection. Further studies to assess the effects of group III mGlu receptor activation on glial responses *in vivo* are clearly warranted to fully gauge the contribution of a neuroinflammatory mechanism to mediate functional neuroprotection.

Collectively, the findings presented in this thesis suggest that, of the group III mGlu receptors investigated, mGlu4 offers the most potential as a promising target for establishing disease modification in PD. It is likely the profound neuroprotective effect mediated by mGlu4 with respect to mGlu7 and to a lesser degree mGlu8 is due



to its greater expression along the subthalamonigral tract. However, an additional factor is that the lack of efficacious, selective pharmacological agents for mGlu7 and mGlu8 receptors has likely contributed to this outcome and a role at least for mGlu7 should not be underestimated at this stage. To assess the clinical utility of selectively targeting mGlu7 receptors, compounds demonstrating greater efficacy and selectivity are eagerly awaited. Furthermore once this goal is achieved, an additional possibility may be that a maximal neuroprotective effect requires activation of more than one group III mGlu receptor subtypes. A similar parallel can be drawn with the neuroprotective effects of nicotine in 6-OHDA lesioned rats, whereby nicotine itself shows the ability to preserve striatal dopamine and motor function, yet individual nicotine receptor subtype agonists could not (Visanji *et al.*, 2006). Future studies should therefore consider the possibility of using a cocktail of group III mGlu receptor subtype selective ligands.

However, for the immediate future, the first objective must be to move away from the current experimental design in initiating treatment prior to lesion, which demonstrates *in vivo* proof of concept yet does not reflect the timing of drug intervention in a clinical setting. The need to follow this paradigm is an attempt to account for the rapid degeneration following 6-OHDA insult however it is clearly a far reflection of the ongoing progressive degenerative process in PD patients and remains a severe limitation of this model. Therefore, future studies must seek to investigate the potential of targeting group III mGlu receptors whereby treatment can be delayed until after the lesion. This will require a more progressive lesion such as that obtained in the striatal 6-OHDA model which leads to nigral cell loss over a period of weeks rather than days.

In the long-term it will also be important to investigate the neuroprotective potential of group III mGlu receptor ligands in a model which leads to the formation of Lewy bodies. In this regard, the 6-OHDA model shares a common failing with many other animal models of PD in failing to mimic this pathological hallmark. Whilst the exact role of Lewy bodies remains to be established, drugs to reduce aggregate formation are considered a potential future strategy for treating PD and animal models must be devised to successfully mimic this pathological phenomenon. Early indications

suggested the proteasomal models such as PSI had strong face validity in this respect, demonstrating progressive loss of nigrostriatal neurones, onset of motor disability and crucially proteinous inclusions relating to Lewy bodies. Unfortunately PSI appears to be highly variable proving difficult to reproduce between laboratories which is hugely frustrating since this could potentially be a valuable model in which to test neuroprotective strategies, but nothing has so far appeared in the literature to this effect. It is a similar story with regard to the genetic models which successfully mimic the mitochondrial dysfunction and subtle abnormalities in dopaminergic transmission consistent with PD, yet fail to replicate nigral pathology. However, genetic studies will no doubt tell us more about the pathology of PD which can be implemented in future improved models. This is urgently awaited considering the fact no translation from animal models of PD into a clinically proven neuroprotective or disease-modifying strategy has been demonstrated to date.

Numerous potentially neuroprotective compounds from a wide range of pharmacological classes that have shown efficacy in rodent and primate models have worryingly *all* proven to be ineffective in the clinic. Dopamine agonists, such as ropinirole and pramipexole, have been reported to be neuroprotective in a range of animals models of PD, including the MPTP treated mouse and 6-OHDA lesioned rat yet there is no clinical evidence to support such effects. Drugs that modify cell death cascades leading to apoptosis have shown to be highly effective in preventing the toxicity of a range of toxins in animal models of PD but have later proved to be inactive in subsequent clinical trials. In fact many potentially neuroprotective compounds ranging from glutamate receptor antagonists, antioxidants, neurotrophic factors and anti-inflammatory agents have all been identified in rodent and primate models, yet none have proved effective in man. It is therefore difficult to see how proceeding with more of the same is going to lead to a neuroprotective strategy for PD.

So where does the problem lie? It may be that selective destruction of dopaminergic neurones through toxin use provides excellent animal models for testing the effects of symptomatic treatments but does not reflect the pathogenic events occurring in man. Indeed, the cause or causes of PD at the molecular level remain unclear, so the target

for neuroprotective therapy is unknown. The animal models of PD currently employed reflect current understanding on the pathogenesis of PD but this thinking may actually be incorrect.

An alternative suggestion may be that the animal models are indeed providing the correct answer but the subsequent clinical trials are at fault. The number of dose levels used in man is limited and may not result in the concentrations reached in the brain of animals. More importantly, it is very likely that there are multiple pathogenic events leading to PD and these vary between individuals. While clinical trials continue to be based on large patient groups of mixed pathology, it may be impossible to detect disease modification that affects small numbers of those receiving active treatment against a background demonstrating lack of effect in most. Therefore considerable attention needs to be given to sub-typing patients with PD based on genetics or biomarkers to avoid further oversight.

Perhaps the greatest limitation hindering the development of a neuroprotective therapy in PD is the identification of a trial design and an outcome measure/biomarker that is not confounded by possible symptomatic or pharmacological effects of the study, but accurately reflects the underlying disease state. It may be necessary to reconsider the study designs and outcome measures that have been used in PD trials aimed at detecting disease modification to accomplish this goal. To date, studies have primarily included patients early in the course of PD using motor measures such as the UPDRS score as the primary outcome measure. However, UPDRS scores are readily confounded by any symptomatic effect of putative neuroprotective agents. Furthermore, it is becoming increasingly evident that long-term disability in PD patients is not so much associated with the classic motor features of the disease (rigidity, bradykinesia, and tremor), which can generally be controlled with symptomatic medications, but with the complex motor and non-motor problems that develop with advancing PD which do not respond to dopaminergic therapies. Indeed, postural instability and freezing (with associated falls and fractures), psychiatric disturbances (hallucinations and depression), and cognitive impairment (dementia) are the major causes of disability in advanced PD patients and the primary reasons for institutionalization. Few trials aimed at disease modification

have focused on the prevention of this type of accumulated disability. It may appear a far stretch to go from addressing mechanisms responsible for neurodegeneration to developing therapies that prevent these generic types of disability, however, the ultimate aim of a neuroprotective treatment is precisely the prevention of these problems.

In the context of this thesis, considering the lack of translation and numerous challenges in store for any ‘hit to lead’, it is far too early to say at this stage whether activation of group III mGlu receptors offer any degree of long-term neuroprotective potential in the treatment of PD. It is essential far more improved, systemically active drugs become available to assess the long-term effectiveness of targeting group III mGlu receptors. This will clarify whether receptor desensitization will have a bearing on the long-term utility of this approach. Certainly many of the responses reported to date with broad spectrum agonists, ranging from electrophysiological studies *in vitro* through to the *in vivo* studies reported in this thesis demonstrate that responses tail off at higher concentrations. These observations are likely a result of receptor desensitization since the broad spectrum agonist L-AP4 has been shown to induce internalisation of at least two of the group III mGlu receptor subtypes (mGlu4 and mGlu7 receptors) as previously discussed in Chapter 3. Targeting the allosteric site of the receptor however is likely to reduce the chance of desensitisation becoming a problem. Indeed from the studies conducted in Chapter 4 using the mGlu4 PAM VU0155041, no such tailing off effect was observed and may help to explain the recent emphasis on developing mGlu4 receptor PAMs rather than the classical orthosteric agonists (Niswender *et al.*, 2008; Engers *et al.*, 2009; Williams *et al.*, 2009).

As of yet, investigations in the group III mGlu receptor field have still not progressed into the final preclinical model of PD, the MPTP primate. This model is the most clinically relevant to monitor the long-term effectiveness of promising agents and such studies will provide a valuable indication into the clinical potential of targeting group III mGlu receptors in PD as well as revealing any unforeseen side effects that may arise from this approach. This is particularly important given that attempts to target glutamatergic transmission via ionotropic glutamate receptor antagonists, was

predicted to lead to unacceptable cognitive and psychomimetic adverse effects. It will therefore be essential to determine if group III mGlu receptors may achieve such inhibition of glutamatergic transmission without the occurrence of similar side effects. Recent clinical trials conducted with agonists of the closely related group II mGlu receptors in patients with schizophrenia have reported good tolerance and crucially no major treatment-related adverse effects (Patil *et al.*, 2007). Whilst the outcome of longer duration clinical trials is still awaited, these earliest observations of targeting group II mGlu receptors in humans provide hope that drugs targeting group III mGlu receptors may prove to be equally well tolerated and devoid of unwanted adverse effects. Nonetheless, the challenge of producing ‘hit-to-lead’ compounds for group III mGlu receptors with the necessary systemic activity may still hinder the translational progress in this area.

In closing, it is important to turn our attention back to the advances achieved in this thesis. From what begun as a hypothetical proposition, the results presented here provide early encouragement to the potential of targeting group III mGlu receptors to provide disease modification in PD. In particular, these findings demonstrate mGlu4 but also mGlu7 can provide functional neuroprotection in an animal model of PD which likely attributes to a reduction in glutamate release from subthalamonigral terminals. Selectively targeting group III mGlu receptors therefore warrants considerable future investigation in additional pre-clinical models of parkinsonism such as the MPTP-treated primate, in order to elucidate their likely long-term neuroprotective potential in the treatment of PD.

## References

- ABELIOVICH, A., SCHMITZ, Y., FARINAS, I., CHOI-LUNDBERG, D., HO, W. H., CASTILLO, P. E., SHINSKY, N., VERDUGO, J. M., ARMANINI, M., RYAN, A., HYNES, M., PHILLIPS, H., SULZER, D., & ROSENTHAL, A. (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25(1), 239-252.
- AHLSKOG, J. E. & MUENTER, M. D. Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. (2001). *Mov Disord.* 16(3), 448-458.
- AIRAS, J. M., BETZ, H., & EL, FAR O. (2001). PKC phosphorylation of a conserved serine residue in the C-terminus of group III metabotropic glutamate receptors inhibits calmodulin binding. *FEBS Lett.* 494(1-2), 60-63.
- ALAGARSAMY, S., MARINO, M. J., ROUSE, S. T., GEREAU, R. W., HEINEMANN, S. F., & CONN, P. J. (1999). Activation of NMDA receptors reverses desensitization of mGluR5 in native and recombinant systems. *Nat.Neurosci.* 2(3), 234-240.
- ALAN, M., SCHMIDT, W.J. (2004). L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone-treated rats. *Behav.Brain. Res.* 153, 439-446.
- ALBANESE, A. & COLOSIMO, C. Dihydroergocriptine in Parkinson's disease: clinical efficacy and comparison with other dopamine agonists. (2003). *Acta Neurol.Scand.* 107(5), 349-355.
- ALBIN, R., YOUNG, A. & PENNEY J. (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci.* 12, 366-375.
- ALEXANDER, G. E., DELONG, M. R., & STRICK, P. L. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu.Rev.Neurosci.* 9, 357-381.
- ALLERS, K. A., BERGSTROM, D. A., GHAZI, L. J., KREISS, D. S., & WALTERS, J. R. (2005). MK801 and amantadine exert different effects on subthalamic neuronal activity in a rodent model of Parkinson's disease. *Exp.Neurol.* 191(1), 104-118.
- ALVAREZ, L., MACIAS, R., LOPEZ, G., ALVAREZ, E., PAVON, N., RODRIGUEZ-OROZ, M. C., JUNCOS, J. L., MARAGOTO, C., GURIDI, J., LITVAN, I., TOLOSA, E. S., KOLLER, W., VITEK, J., DELONG, M. R., & OBESO, J. A. (2005). Bilateral subthalamotomy in Parkinson's disease: initial and long-term response. *Brain* 128(Pt 3), 570-583.
- ANDERSON, V. C., BURCHIEL, K. J., HOGARTH, P., FAVRE, J., & HAMMERSTAD, J. P. (2005). Pallidal vs subthalamic nucleus deep brain stimulation in Parkinson disease. *Arch.Neurol.* 62(4), 554-560.

ARONICA, E., GORTER, J. A., IJLST-KEIZERS, H., ROZEMULLER, A. J., YANKAYA, B., LEENSTRA, S., & TROOST, D. (2003). Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. *Eur.J.Neurosci.* 17(10), 2106-2118.

ASCHERIO, A., CHEN, H., WEISSKOPF, M.G., O'REILLY, E., MCCULLOUGH, M.L., CALLE, E.E., SCHWARZSCHILD, M.A., THUN, M.J (2006). Pesticide exposure and risk for Parkinson's disease. *Ann. Neurol.* 60, 197-203.

ASCHERIO, A., ZHANG, S. M., HERNAN, M. A., KAWACHI, I., COLDITZ, G. A., SPEIZER, F. E., & WILLETT, W. C. (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Ann.Neurol.* 50(1), 56-63.

AUSTIN, P. J., BETTS, M. J., BROADSTOCK, M., O'NEILL, M. J., MITCHELL, S. N., & DUTY, S. (2010). Symptomatic and neuroprotective effects following activation of nigral group III metabotropic glutamate receptors in rodent models of Parkinson's disease. *Br.J.Pharmacol.* 160(7), 1741-1753.

BANATI, R. B., DANIEL, S. E., & BLUNT, S. B. (1998). Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. *Mov Disord.* 13(2), 221-227.

BARA-JIMENEZ, W., SHERZAI, A., DIMITROVA, T., FAVIT, A., BIBBIANI, F., GILLESPIE, M., MORRIS, M. J., MOURADIAN, M. M., & CHASE, T. N. (2003). Adenosine A(2A) receptor antagonist treatment of Parkinson's disease. *Neurology* 61(3), 293-296.

BATTAGLIA, G., BUSCETI, C. L., MOLINARO, G., BIAGIONI, F., TRAFICANTE, A., NICOLETTI, F., & BRUNO, V. (2006). Pharmacological activation of mGlu4 metabotropic glutamate receptors reduces nigrostriatal degeneration in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J.Neurosci.* 26(27), 7222-7229.

BECKSTEAD, R. M. & KERSEY, K. S. (1985). Immunohistochemical demonstration of differential substance P-, met-enkephalin-, and glutamic-acid-decarboxylase-containing cell body and axon distributions in the corpus striatum of the cat. *J.Comp Neurol.* 232(4), 481-498.

BEDARD, A., GRAVEL, C., & PARENT, A. (2006). Chemical characterization of newly generated neurons in the striatum of adult primates. *Exp.Brain Res.* 170(4), 501-512.

BERENDSE, H. W., GALIS-DE, GRAAF Y., & GROENEWEGEN, H. J. (1992). Topographical organization and relationship with ventral striatal compartments of prefrontal corticostriatal projections in the rat. *J.Comp Neurol.* 316(3), 314-347.

BERGINK, V. & WESTENBERG, H. G. (2005). Metabotropic glutamate II receptor agonists in panic disorder: a double blind clinical trial with LY354740. *Int.Clin.Psychopharmacol.* 20(6), 291-293.

- BERGMAN, H., WICHMANN, T., KARMON, B., & DELONG, M. R. (1994). The primate subthalamic nucleus. II. Neuronal activity in the MPTP model of parkinsonism. *J.Neurophysiol.* 72(2), 507-520.
- BERNHEIMER, H., BIRKMAYER, W., HORNYKIEWICZ, O., JELLINGER, K., SEITELBERGER, F. (1973). Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J. Neurol. Sci.* 20, 415-455
- BESONG, G., BATTAGLIA, G., D'ONOFRIO, M., DI, MARCO R., NGOMBA, R. T., STORTO, M., CASTIGLIONE, M., MANGANO, K., BUSCETI, C. L., NICOLETTI, F. R., BACON, K., TUSCHE, M., VALENTI, O., CONN, P. J., BRUNO, V., & NICOLETTI, F. (2002). Activation of group III metabotropic glutamate receptors inhibits the production of RANTES in glial cell cultures. *J.Neurosci.* 22(13), 5403-5411.
- BETARBET, R., SHERER, T. B., & GREENAMYRE, J. T. (2002). Animal models of Parkinson's disease. *Bioessays* 24(4), 308-318.
- BETARBET, R., SHERER, T. B., MACKENZIE, G., GARCIA-OSUNA, M., PANOV, A. V., & GREENAMYRE, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat.Neurosci.* 3(12), 1301-1306.
- BETARBET, R., TURNER, R., CHOCKKAN, V., DELONG, M. R., ALLERS, K. A., WALTERS, J., LEVEY, A. I., & GREENAMYRE, J. T. (1997). Dopaminergic neurons intrinsic to the primate striatum. *J.Neurosci.* 17(17), 6761-6768.
- BEYER, M. K., HERLOFSON, K., ARSLAND, D., & LARSEN, J. P. (2001). Causes of death in a community-based study of Parkinson's disease. *Acta Neurol.Scand.* 103(1), 7-11.
- BEZARD, E., IMBERT, C., & GROSS, C. E. (1998). Experimental models of Parkinson's disease: from the static to the dynamic. *Rev.Neurosci.* 9(2), 71-90.
- BILLINGS, L. M. & MARSHALL, J. F. (2004). Glutamic acid decarboxylase 67 mRNA regulation in two globus pallidus neuron populations by dopamine and the subthalamic nucleus. *J.Neurosci.* 24(12), 3094-3103.
- BJORKLUND, L. M., SANCHEZ-PERNAUTE, R., CHUNG, S., ANDERSSON, T., CHEN, I. Y., MCNAUGHT, K. S., BROWNELL, A. L., JENKINS, B. G., WAHLESTEDT, C., KIM, K. S., & ISACSON, O. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc.Natl.Acad.Sci.U.S.A.* 99(4), 2344-2349.
- BLANDINI, F., NAPPI, G., & GREENAMYRE, J. T. (2001). Subthalamic infusion of an NMDA antagonist prevents basal ganglia metabolic changes and nigral degeneration in a rodent model of Parkinson's disease. *Ann.Neurol.* 49(4), 525-529.
- BLANDINI, F., NAPPI, G., TASSORELLI, C., & MARTIGNONI, E. (2000). Functional changes of the basal ganglia circuitry in Parkinson's disease. *Prog.Neurobiol.* 62(1), 63-88.



- BLUM, D., TORCH, S., LAMBENG, N., NISSOU, M., BENABID, A. L., SADOUL, R., & VERNA, J. M. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog.Neurobiol.* 65(2), 135-172.
- BOEVE, B. F., SILBER, M. H., SAPER, C. B., FERMAN, T. J., DICKSON, D. W., PARISI, J. E., BENARROCH, E. E., AHLISKOG, J. E., SMITH, G. E., CASELLI, R. C., TIPPMAN-PEIKERT, M., OLSON, E. J., LIN, S. C., YOUNG, T., WSZOLEK, Z., SCHENCK, C. H., MAHOWALD, M. W., CASTILLO, P. R., DEL, TREDICI K., & BRAAK, H. (2007). Pathophysiology of REM sleep behaviour disorder and relevance to neurodegenerative disease. *Brain* 130(Pt 11), 2770-2788.
- BOKA, G., ANGLADE, P., WALLACH, D., JAVOY-AGID, F., AGID, Y., & HIRSCH, E. C. (1994). Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci.Lett.* 172(1-2), 151-154.
- BOLAM, J. P., HANLEY, J. J., BOOTH, P. A., & BEVAN, M. D. (2000). Synaptic organisation of the basal ganglia. *J.Anat.* 196 ( Pt 4), 527-542.
- BONIFATI, V., RIZZU, P., VAN BAREN, M. J., SCHAAP, O., BREEDVELD, G. J., KRIEGER, E., DEKKER, M. C., SQUITIERI, F., IBANEZ, P., JOOSSE, M., VAN DONGEN, J. W., VANACORE, N., VAN SWIETEN, J. C., BRICE, A., MECO, G., VAN DUIJN, C. M., OOSTRA, B. A., & HEUTINK, P. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299(5604), 256-259.
- BONSI, P., CUOMO, D., PICCONI, B., SCIAMANNA, G., TSCHERTER, A., TOLU, M., BERNARDI, G., CALABRESI, P., & PISANI, A. (2007). Striatal metabotropic glutamate receptors as a target for pharmacotherapy in Parkinson's disease. *Amino.Acids.* 32(2), 189-195.
- BOULWARE, M. I., WEICK, J. P., BECKLUND, B. R., KUO, S. P., GROTH, R. D., & MERMELSTEIN, P. G. (2005). Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. *J.Neurosci.* 25(20), 5066-5078.
- BOVE, J., SERRATS, J., MENGOD, G., CORTES, R., TOLOSA, E., & MARIN, C. (2005). Neuroprotection induced by the adenosine A2A antagonist CSC in the 6-OHDA rat model of parkinsonism: effect on the activity of striatal output pathways. *Exp.Brain Res.* 165(3), 362-374.
- BRAAK, H., DEL, TREDICI K., RUB, U., DE VOS, R. A., JANSEN STEUR, E. N., & BRAAK, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol.Aging* 24(2), 197-211.
- BRADLEY, S. R., MARINO, M. J., WITTMANN, M., ROUSE, S. T., AWAD, H., LEVEY, A. I., & CONN, P. J. (2000). Activation of group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia Nigra pars reticulata. *J.Neurosci.* 20(9), 3085-3094.

BRADLEY, S. R., STANDAERT, D. G., LEVEY, A. I., & CONN, P. J. (1999). Distribution of group III mGluRs in rat basal ganglia with subtype-specific antibodies. *Ann.N.Y.Acad.Sci.* 868, 531-534.

BREIDERT, T., CALLEBERT, J., HENEKA, M. T., LANDRETH, G., LAUNAY, J. M., & HIRSCH, E. C. (2002). Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. *J.Neurochem.* 82(3), 615-624.

BREIT, S., LESSMANN, L., UNTERBRINK, D., POPA, R. C., GASSER, T., & SCHULZ, J. B. (2006). Lesion of the pedunculopontine nucleus reverses hyperactivity of the subthalamic nucleus and substantia nigra pars reticulata in a 6-hydroxydopamine rat model. *Eur.J.Neurosci.* 24(8), 2275-2282.

BREIT, S., SCHULZ, J. B., & BENABID, A. L. (2004). Deep brain stimulation. *Cell Tissue Res.* 318(1), 275-288.

BREYSSE, N., BAUNEZ, C., SPOOREN, W., GASPARINI, F., & AMALRIC, M. (2002). Chronic but not acute treatment with a metabotropic glutamate 5 receptor antagonist reverses the akinetic deficits in a rat model of parkinsonism. *J.Neurosci.* 22(13), 5669-5678.

BROADSTOCK, M., AUSTIN, P., BETTS, M., & DUTY, S. (2011). Antiparkinsonian potential of targeting group III metabotropic glutamate receptor subtypes in the rodent substantia nigra pars reticulata. *Br.J.Pharmacol.*

BRODBECK, RM., SOTTY, F., PU, X., UBERTI, M., ISAAC, LK., SCHULENBURG, T. (2010). Characterisation of AMN082: A rich pharmacology which does not appear to include a measurable stimulation of mGlu7 receptor. *SFN Abstract*, Nov 2010: 159.23

BROER, S. & BROOKES, N. (2001). Transfer of glutamine between astrocytes and neurons. *J.Neurochem.* 77(3), 705-719.

BRONSTEIN, D. M., PEREZ-OTANO, I., SUN, V., MULLIS SAWIN, S. B., CHAN, J., WU, G. C., HUDSON, P. M., KONG, L. Y., HONG, J. S., & MCMILLIAN, M. K. (1995). Glia-dependent neurotoxicity and neuroprotection in mesencephalic cultures. *Brain Res.* 704(1), 112-116.

BROOKS, D. J. & SAGAR, H. (2003). Entacapone is beneficial in both fluctuating and non-fluctuating patients with Parkinson's disease: a randomised, placebo controlled, double blind, six month study. *J.Neurol.Neurosurg.Psychiatry.* 74(8), 1071-1079.

BRUNO, V., BATTAGLIA, G., CASABONA, G., COPANI, A., CACIAGLI, F., & NICOLETTI, F. (1998). Neuroprotection by glial metabotropic glutamate receptors is mediated by transforming growth factor-beta. *J.Neurosci.* 18(23), 9594-9600.

BRUNO, V., BATTAGLIA, G., COPANI, A., D'ONOFRIO, M., DI, IORIO P., DE, BLASI A., MELCHIORRI, D., FLOR, P. J., & NICOLETTI, F. (2001). Metabotropic

glutamate receptor subtypes as targets for neuroprotective drugs. *J.Cereb.Blood Flow Metab* 21(9), 1013-1033.

BUKHATWA, S., ZENG, B.Y., ROSE, S., JENNER, P. (2010). The effects of dose and route of administration of PSI on behavioural and biochemical indices of neuronal degeneration in the rat brain. *Brain. Res.* 1354, 236-242.

BURNASHEV, N., ZHOU, Z., NEHER, E., & SAKMANN, B. (1995). Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes. *J.Physiol* 485 ( Pt 2), 403-418.

BURNS, R.S., CHIUEH, C.C., MARKEY, S.P., EBERT, M.H., JACOBOWITZ, D.M., KOPIN, I.J. (1983). A primate model of Parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Nat. Acad. Sci. USA.* 80, 4546-4550.

CAI, Z., SAUGSTAD, J. A., SORENSEN, S. D., CIOMBOR, K. J., ZHANG, C., SCHAFFHAUSER, H., HUBALEK, F., POHL, J., DUVOISIN, R. M., & CONN, P. J. (2001). Cyclic AMP-dependent protein kinase phosphorylates group III metabotropic glutamate receptors and inhibits their function as presynaptic receptors. *J.Neurochem.* 78(4), 756-766.

CALABRESI, P., PISANI, A., MERCURI, N. B., & BERNARDI, G. (1996). The corticostriatal projection: from synaptic plasticity to dysfunctions of the basal ganglia. *Trends Neurosci.* 19(1), 19-24.

CALON, F., GRONDIN, R., MORISSETTE, M., GOULET, M., BLANCHET, P. J., DI, PAOLO T., & BEDARD, P. J. (2000). Molecular basis of levodopa-induced dyskinesias. *Ann.Neurol.* 47(4 Suppl 1), S70-S78.

CAMPUSANO, J. M., ABARCA, J., FORRAY, M. I., GYSLING, K., & BUSTOS, G. (2002). Modulation of dendritic release of dopamine by metabotropic glutamate receptors in rat substantia nigra. *Biochem.Pharmacol.* 63(7), 1343-1352.

CANTERAS, N. S., SHAMMAH-LAGNADO, S. J., SILVA, B. A., & RICARDO, J. A. (1990). Afferent connections of the subthalamic nucleus: a combined retrograde and anterograde horseradish peroxidase study in the rat. *Brain Res.* 513(1), 43-59.

CARLSSON, A., LINDQVIST, M., MAGNUSSON, T. (1957). 3,4,-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature.* 180:1200

CARMAN, L. S., GAGE, F. H., & SHULTS, C. W. (1991). Partial lesion of the substantia nigra: relation between extent of lesion and rotational behavior. *Brain Res.* 553(2), 275-283.

CARR, J., DE, LA FUENTE-FERNANDEZ, SCHULZER, M., MAK, E., CALNE, S. M., & CALNE, D. B. (2003). Familial and sporadic Parkinson's disease usually display the same clinical features. *Parkinsonism.Relat Disord.* 9(4), 201-204.

- CARTER, C. J. (1982). Topographical distribution of possible glutamatergic pathways from the frontal cortex to the striatum and substantia nigra in rats. *Neuropharmacology* 21(5), 379-383.
- CARTMELL, J. & SCHOEPP, D. D. (2000). Regulation of neurotransmitter release by metabotropic glutamate receptors. *J.Neurochem.* 75(3), 889-907.
- CHANG, H. T. (1988). Dopamine-acetylcholine interaction in the rat striatum: a dual-labeling immunocytochemical study. *Brain Res.Bull.* 21(2), 295-304.
- CHEN, J. F., XU, K., PETZER, J. P., STAAL, R., XU, Y. H., BEILSTEIN, M., SONSALLA, P. K., CASTAGNOLI, K., CASTAGNOLI, N., JR., &
- CHIBA, K., TREVOR, A., CASTAGNOLI, N. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem. Biophys. Res. Commun.* 120, 574-578.
- SCHWARZSCHILD, M. A. (2001). Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J.Neurosci.* 21(10), RC143.
- CHOI, J., SULLARDS, M. C., OLZMANN, J. A., REES, H. D., WEINTRAUB, S. T., BOSTWICK, D. E., GEARING, M., LEVEY, A. I., CHIN, L. S., & LI, L. (2006). Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. *J.Biol.Chem.* 281(16), 10816-10824.
- CHRISTOFFERSEN, C. L. & MELTZER, L. T. (1995). Evidence for N-methyl-D-aspartate and AMPA subtypes of the glutamate receptor on substantia nigra dopamine neurons: possible preferential role for N-methyl-D-aspartate receptors. *Neuroscience* 67(2), 373-381.
- CHUNG, K. K., ZHANG, Y., LIM, K. L., TANAKA, Y., HUANG, H., GAO, J., ROSS, C. A., DAWSON, V. L., & DAWSON, T. M. (2001). Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat.Med.* 7(10), 1144-1150.
- CICCHETTI, F., BROWNELL, A. L., WILLIAMS, K., CHEN, Y. I., LIVNI, E., & ISACSON, O. (2002). Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. *Eur.J.Neurosci.* 15(6), 991-998.
- CONN, P. J. & PIN, J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annu.Rev.Pharmacol.Toxicol.* 37, 205-237.
- CORTI, C., ALDEGHERI, L., SOMOGYI, P., & FERRAGUTI, F. (2002). Distribution and synaptic localisation of the metabotropic glutamate receptor 4 (mGluR4) in the rodent CNS. *Neuroscience* 110(3), 403-420.
- CORTI, C., RESTITUITO, S., RIMLAND, J. M., BRABET, I., CORSI, M., PIN, J. P., & FERRAGUTI, F. (1998). Cloning and characterization of alternative mRNA

forms for the rat metabotropic glutamate receptors mGluR7 and mGluR8. *Eur.J.Neurosci.* 10(12), 3629-3641.

COSTELLO, S., COCKBURN, M., BRONSTEIN, J., ZHANG, X., RITZ, B. (2009). Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. *Am. J. Epidemiol.* 169, 919-926.

CROSSMAN, A. R., MITCHELL, I. J., & SAMBROOK, M. A. (1985). Regional brain uptake of 2-deoxyglucose in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in the macaque monkey. *Neuropharmacology* 24(6), 587-591.

CROSSMAN, A. R., PEGGS, D., BOYCE, S., LUQUIN, M. R., & SAMBROOK, M. A. (1989). Effect of the NMDA antagonist MK-801 on MPTP-induced parkinsonism in the monkey. *Neuropharmacology* 28(11), 1271-1273.

CROUCHER, M. J., THOMAS, L. S., AHMADI, H., LAWRENCE, V., & HARRIS, J. R. (2001). Endogenous sulphur-containing amino acids: potent agonists at presynaptic metabotropic glutamate autoreceptors in the rat central nervous system. *Br.J.Pharmacol.* 133(6), 815-824.

DAMIER, P., HIRSCH, E. C., AGID, Y., & GRAYBIEL, A. M. (1999). The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain* 122 ( Pt 8), 1437-1448.

DATLA, K. P., MURRAY, H. E., PILLAI, A. V., GILLIES, G. E., & DEXTER, D. T. (2003). Differences in dopaminergic neuroprotective effects of estrogen during estrous cycle. *Neuroreport* 14(1), 47-50.

DAVIS, G.C., WILLIAMS, A.C., MARKEY, S.P., EBERT, M.H., CAINE, E.D., REICHERT, C.M., KOPIN, I.J. (1979). Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. *Psychiatry. Res.* 1, 249-254.

DAWSON, V. L. & DAWSON, T. M. (1998). Nitric oxide in neurodegeneration. *Prog.Brain Res.* 118:215-29., 215-229.

DE WAARD M., LIU, H., WALKER, D., SCOTT, V. E., GURNETT, C. A., & CAMPBELL, K. P. (1997). Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature* 385(6615), 446-450.

DEHMER, T., HENEKA, M. T., SASTRE, M., DICHGANS, J., & SCHULZ, J. B. (2004). Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B alpha induction and block of NF kappa B and iNOS activation. *J.Neurochem.* 88(2), 494-501.

DEUMENS, R., BLOKLAND, A., & PRICKAERTS, J. (2002). Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp.Neurol.* 175(2), 303-317.

DEV, K. K., NAKANISHI, S., & HENLEY, J. M. (2001). Regulation of mglu(7) receptors by proteins that interact with the intracellular C-terminus. *Trends Pharmacol.Sci.* 22(7), 355-361.

DEXTER, D. T., WELLS, F. R., LEES, A. J., AGID, F., AGID, Y., JENNER, P., & MARSDEN, C. D. (1989). Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. *J.Neurochem.* 52(6), 1830-1836.

DHEEN, S. T., KAUR, C., & LING, E. A. (2007). Microglial activation and its implications in the brain diseases. *Curr.Med.Chem.* 14(11), 1189-1197.

DI LORETO S., FLORIO, T., & SCARNATI, E. (1992). Evidence that non-NMDA receptors are involved in the excitatory pathway from the pedunculopontine region to nigrostriatal dopaminergic neurons. *Exp.Brain Res.* 89(1), 79-86.

DICKSON, D. W., FUJISHIRO, H., ORR, C., DELLEDONNE, A., JOSEPHS, K. A., FRIGERIO, R., BURNETT, M., PARISI, J. E., KLOS, K. J., & AHLISKOG, J. E. (2009). Neuropathology of non-motor features of Parkinson disease. *Parkinsonism.Relat Disord.* 15 Suppl 3, S1-S5.

DOBLE, A. (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol.Ther.* 81(3), 163-221.

DUDA, J.E., GIASSEN, B.I., CHEN, Q., GUR, T.L., HURTIG, H., STERN, M.B., GOLLOMP, S.M., ISCHIROPOULOS, H., LEE, V.M., TROJANOWSKI, J.Q. (2000). Widespread nitration of pathological inclusions in neurodegenerative synucleinopathies. *Am. J. Pathol.* 157, 1439-1445.

DUNNETT, S. B. & BJORKLUND, A. (1999). Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature* 399(6738 Suppl), A32-A39.

DUTY, S. (2010). Therapeutic potential of targeting group III metabotropic glutamate receptors in the treatment of Parkinson's disease. *Br.J.Pharmacol.* 161(2), 271-287.

DUTY, S. & JENNER, P. (2011). Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease. *Br.J.Pharmacol.*

EAST, S. P., BAMFORD, S., DIETZ, M. G., EICKMEIER, C., FLEGG, A., FERGER, B., GEMKOW, M. J., HEILKER, R., HENGERER, B., KOTEY, A., LOKE, P., SCHANZLE, G., SCHUBERT, H. D., SCOTT, J., WHITTAKER, M., WILLIAMS, M., ZAWADZKI, P., & GERLACH, K. (2010). An orally bioavailable positive allosteric modulator of the mGlu4 receptor with efficacy in an animal model of motor dysfunction. *Bioorg.Med.Chem.Lett.* 20(16), 4901-4905.

EHRINGER, H. & HORNYKIEWICZ, O. (1960). [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system]. *Klin.Wochenschr.* 38, 1236-1239.

EL, FAR O., AIRAS, J., WISCHMEYER, E., NEHRING, R. B., KARSCHIN, A., & BETZ, H. (2000). Interaction of the C-terminal tail region of the metabotropic

glutamate receptor 7 with the protein kinase C substrate PICK1. *Eur.J.Neurosci.* 12(12), 4215-4221.

ELEZGARAI, I., BENITEZ, R., MATEOS, J. M., LAZARO, E., OSORIO, A., AZKUE, J. J., BILBAO, A., LINGENHOEHL, K., VAN DER, PUTTEN H., HAMPSON, D. R., KUHN, R., KNOPFEL, T., & GRANDES, P. (1999). Developmental expression of the group III metabotropic glutamate receptor mGluR4a in the medial nucleus of the trapezoid body of the rat. *J.Comp Neurol.* 411(3), 431-440.

EMRE, M., AARSLAND, D., ALBANESE, A., BYRNE, E. J., DEUSCHL, G., DE DEYN, P. P., DURIF, F., KULISEVSKY, J., VAN, LAAR T., LEES, A., POEWE, W., ROBILLARD, A., ROSA, M. M., WOLTERS, E., QUARG, P., TEKIN, S., & LANE, R. (2004). Rivastigmine for dementia associated with Parkinson's disease. *N.Engl.J.Med.* 351(24), 2509-2518.

ENGERS, D. W., GENTRY, P. R., WILLIAMS, R., BOLINGER, J. D., WEAVER, C. D., MENON, U. N., CONN, P. J., LINDSLEY, C. W., NISWENDER, C. M., & HOPKINS, C. R. (2010). Synthesis and SAR of novel, 4-(phenylsulfamoyl)phenylacetamide mGlu4 positive allosteric modulators (PAMs) identified by functional high-throughput screening (HTS). *Bioorg.Med.Chem.Lett.* 20(17), 5175-5178.

ENZ, R. & CROCI, C. (2003). Different binding motifs in metabotropic glutamate receptor type 7b for filamin A, protein phosphatase 1C, protein interacting with protein kinase C (PICK) 1 and syntenin allow the formation of multimeric protein complexes. *Biochem.J.* 372(Pt 1), 183-191.

ERDO, F., BERZSENYI, P., & ANDRASI, F. (2005). The AMPA-antagonist talampanel is neuroprotective in rodent models of focal cerebral ischemia. *Brain Res.Bull.* 66(1), 43-49.

ESLAMBOLI, A., GEORGIEVSKA, B., RIDLEY, R. M., BAKER, H. F., MUZYCZKA, N., BURGER, C., MANDEL, R. J., ANNETT, L., & KIRIK, D. (2005). Continuous low-level glial cell line-derived neurotrophic factor delivery using recombinant adeno-associated viral vectors provides neuroprotection and induces behavioral recovery in a primate model of Parkinson's disease. *J.Neurosci.* 25(4), 769-777.

FABBRINI, G., BARBANTI, P., AURILIA, C., PAULETTI, C., LENZI, G. L., & MECO, G. (2002). Donepezil in the treatment of hallucinations and delusions in Parkinson's disease. *Neurol.Sci.* 23(1), 41-43.

FAULL, R. L. & LAVERTY, R. (1969). Changes in dopamine levels in the corpus striatum following lesions in the substantia nigra. *Exp.Neurol.* 23(3), 332-340.

FAVARON, M., MANEV, H., SIMAN, R., BERTOLINO, M., SZEKELY, A. M., DEERAUSQUIN, G., GUIDOTTI, A., & COSTA, E. (1990). Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. *Proc.Natl.Acad.Sci.U.S.A* 87(5), 1983-1987.

FERRAGUTI, F. & SHIGEMOTO, R. (2006). Metabotropic glutamate receptors. *Cell Tissue Res.* 326(2), 483-504.

FERRANTE, R. J., HANTRAYE, P., BROUILLET, E., & BEAL, M. F. (1999). Increased nitrotyrosine immunoreactivity in substantia nigra neurons in MPTP treated baboons is blocked by inhibition of neuronal nitric oxide synthase. *Brain Res.* 823(1-2), 177-182.

FERRE, S. & FUXE, K. (1992). Dopamine denervation leads to an increase in the intramembrane interaction between adenosine A2 and dopamine D2 receptors in the neostriatum. *Brain Res.* 594(1), 124-130.

FILION, M. & TREMBLAY, L. (1991). Abnormal spontaneous activity of globus pallidus neurons in monkeys with MPTP-induced parkinsonism. *Brain Res.* 547(1), 142-151.

FINK, J. S., WEAVER, D. R., RIVKEES, S. A., PETERFREUND, R. A., POLLACK, A. E., ADLER, E. M., & REPPERT, S. M. (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res.Mol.Brain Res.* 14(3), 186-195.

FINK-JENSEN, A. & MIKKELSEN, J. D. (1989). The striato-entopeduncular pathway in the rat. A retrograde transport study with wheatgerm-agglutinin-horseradish peroxidase. *Brain Res.* 476(1), 194-198.

FOGELSON, N., KUHN, A. A., SILBERSTEIN, P., LIMOUSIN, P. D., HARIZ, M., TROTTENBERG, T., KUPSCH, A., & BROWN, P. (2005). Frequency dependent effects of subthalamic nucleus stimulation in Parkinson's disease. *Neurosci.Lett.* 382(1-2), 5-9.

FONNUM, F., GROFAVA, I., & RINVIK, E. (1978). Origin and distribution of glutamate decarboxylase in the nucleus subthalamicus of the cat. *Brain Res.* 153(2), 370-374.

FORNAI, F., LENZI, P., GESI, M., FERRUCCI, M., LAZZERI, G., BUSCETI, C. L., RUFFOLI, R., SOLDANI, P., RUGGIERI, S., ALESSANDRI, M. G., & PAPARELLI, A. (2003). Fine structure and biochemical mechanisms underlying nigrostriatal inclusions and cell death after proteasome inhibition. *J.Neurosci.* 23(26), 8955-8966.

FORNAI, F., SCHLUTER, O.M., LENZI, P., GESI, M., RUFFOLI, R., FERRUCCI, M., LAZZERI, G., BUSCETI, C.L., PONTARELLI, F., BATTAGLIA, G., PELLEGRINI, A., NICOLETTI, F., RUGGIERI, S., PAPARELLI, A., SUDHOF, T.C. (2005). Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and  $\alpha$ -synuclein. *Proc. Natl. Acad. Sci. USA.* 102, 3413-3418.

FRANCOIS, C., SAVY, C., JAN, C., TANDE, D., HIRSCH, E. C., & YELNIK, J. (2000). Dopaminergic innervation of the subthalamic nucleus in the normal state, in MPTP-treated monkeys, and in Parkinson's disease patients. *J.Comp Neurol.* 425(1), 121-129.



FRANCOIS, C., YELNIK, J., TANDE, D., AGID, Y., & HIRSCH, E. C. (1999). Dopaminergic cell group A8 in the monkey: anatomical organization and projections to the striatum. *J.Comp Neurol.* 414(3), 334-347.

FUJIMOTO, K. & KITA, H. (1993). Response characteristics of subthalamic neurons to the stimulation of the sensorimotor cortex in the rat. *Brain Res.* 609(1-2), 185-192.

GAGNON, J. F., BEDARD, M. A., FANTINI, M. L., PETIT, D., PANISSET, M., ROMPRE, S., CARRIER, J., & MONTPLAISIR, J. (2002). REM sleep behavior disorder and REM sleep without atonia in Parkinson's disease. *Neurology* 59(4), 585-589.

GATEV, P., DARBIN, O., & WICHMANN, T. (2006). Oscillations in the basal ganglia under normal conditions and in movement disorders. *Mov Disord.* 21(10), 1566-1577.

GERHARD, A., PAVESE, N., HOTTON, G., TURKHEIMER, F., ES, M., HAMMERS, A., EGGERT, K., OERTEL, W., BANATI, R. B., & BROOKS, D. J. (2006). In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. *Neurobiol.Dis.* 21(2), 404-412.

GERMAN, D. C., SCHLUSSELBERG, D. S., & WOODWARD, D. J. (1983). Three-dimensional computer reconstruction of midbrain dopaminergic neuronal populations: from mouse to man. *J.Neural Transm.* 57(4), 243-254.

GIASSON, B. I., DUDA, J. E., MURRAY, I. V., CHEN, Q., SOUZA, J. M., HURTIG, H. I., ISCHIROPOULOS, H., TROJANOWSKI, J. Q., & LEE, V. M. (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290(5493), 985-989.

GILL, S. S., PATEL, N. K., HOTTON, G. R., O'SULLIVAN, K., MCCARTER, R., BUNNAGE, M., BROOKS, D. J., SVENDSEN, C. N., & HEYWOOD, P. (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat.Med.* 9(5), 589-595.

GLINKA, Y. Y. & YODIM, M. B. (1995). Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. *Eur.J.Pharmacol.* 292(3-4), 329-332.

GOLDWURM, S., DI, FONZO A., SIMONS, E. J., ROHE, C. F., ZINI, M., CANESI, M., TESEI, S., ZECCHINELLI, A., ANTONINI, A., MARIANI, C., MEUCCI, N., SACILOTTO, G., SIRONI, F., SALANI, G., FERREIRA, J., CHIEN, H. F., FABRIZIO, E., VANACORE, N., DALLA, LIBERA A., STOCCHI, F., DIROMA, C., LAMBERTI, P., SAMPAIO, C., MECO, G., BARBOSA, E., BERTOLI-AVELLA, A. M., BREEDVELD, G. J., OOSTRA, B. A., PEZZOLI, G., & BONIFATI, V. (2005). The G6055A (G2019S) mutation in LRRK2 is frequent in both early and late onset Parkinson's disease and originates from a common ancestor. *J.Med.Genet.* 42(11), e65.

GOMEZ-LAZARO, M., GALINDO, M. F., CONCANNON, C. G., SEGURA, M. F., FERNANDEZ-GOMEZ, F. J., LLECHA, N., COMELLA, J. X., PREHN, J. H., & JORDAN, J. (2008). 6-Hydroxydopamine activates the mitochondrial apoptosis

pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. *J.Neurochem.* 104(6), 1599-1612.

GOOD, P. F., HSU, A., WERNER, P., PERL, D. P., & OLANOW, C. W. (1998). Protein nitration in Parkinson's disease. *J.Neuropathol.Exp.Neurol.* 57(4), 338-342.

GORELL, J. M., JOHNSON, C. C., RYBICKI, B. A., PETERSON, E. L., KORTSHA, G. X., BROWN, G. G., & RICHARDSON, R. J. (1997). Occupational exposures to metals as risk factors for Parkinson's disease. *Neurology* 48(3), 650-658.

GRAHAM, D. G. (1978). Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol.Pharmacol.* 14(4), 633-643.

GRAVELAND, G. A. & DIFIGLIA, M. (1985). The frequency and distribution of medium-sized neurons with indented nuclei in the primate and rodent neostriatum. *Brain Res.* 327(1-2), 307-311.

GRECO, B., LOPEZ, S., VAN DER, PUTTEN H., FLOR, P. J., & AMALRIC, M. (2010). Metabotropic glutamate 7 receptor subtype modulates motor symptoms in rodent models of Parkinson's disease. *J.Pharmacol.Exp.Ther.* 332(3), 1064-1071.

GREENAMYRE, J. T. (2001). Glutamatergic influences on the basal ganglia. *Clin.Neuropharmacol.* 24(2), 65-70.

GREENAMYRE, J. T., ELLER, R. V., ZHANG, Z., OVADIA, A., KURLAN, R., & GASH, D. M. (1994). Antiparkinsonian effects of remacemide hydrochloride, a glutamate antagonist, in rodent and primate models of Parkinson's disease. *Ann.Neurol.* 35(6), 655-661.

GREENE, J. G. & GREENAMYRE, J. T. (1996). Bioenergetics and glutamate excitotoxicity. *Prog.Neurobiol.* 48(6), 613-634.

GROENEWEGEN, H. J., BERENDSE, H. W., & HABER, S. N. (1993). Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. *Neuroscience* 57(1), 113-142.

GRONDIN, R., ZHANG, Z., YI, A., CASS, W. A., MASWOOD, N., ANDERSEN, A. H., ELSBERRY, D. D., KLEIN, M. C., GERHARDT, G. A., & GASH, D. M. (2002). Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. *Brain* 125(Pt 10), 2191-2201.

GUBELLINI, P., PISANI, A., CENTONZE, D., BERNARDI, G., & CALABRESI, P. (2004). Metabotropic glutamate receptors and striatal synaptic plasticity: implications for neurological diseases. *Prog.Neurobiol.* 74(5), 271-300.

GUIGONI, C., DOVERO, S., AUBERT, I., LI, Q., BIOULAC, B. H., BLOCH, B., GUREVICH, E. V., GROSS, C. E., & BEZARD, E. (2005). Levodopa-induced dyskinesia in MPTP-treated macaques is not dependent on the extent and pattern of nigrostriatal lesioning. *Eur.J.Neurosci.* 22(1), 283-287.

- GURIDI, J., HERRERO, M. T., LUQUIN, M. R., GUILLEN, J., RUBERG, M., LAGUNA, J., VILA, M., JAVOY-AGID, F., AGID, Y., HIRSCH, E., & OBESO, J. A. (1996). Subthalamotomy in parkinsonian monkeys. Behavioural and biochemical analysis. *Brain* 119 ( Pt 5), 1717-1727.
- GYGI, S. P., ROCHON, Y., FRANZA, B. R., & AEBERSOLD, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol.Cell Biol.* 19(3), 1720-1730.
- HABER, S. & MCFARLAND, N. R. (2001). The place of the thalamus in frontal cortical-basal ganglia circuits. *Neuroscientist.* 7(4), 315-324.
- HABER, S. N. (2003). The primate basal ganglia: parallel and integrative networks. *J.Chem.Neuroanat.* 26(4), 317-330.
- HADJ, TAHAR A., GREGOIRE, L., DARRE, A., BELANGER, N., MELTZER, L., & BEDARD, P. J. (2004). Effect of a selective glutamate antagonist on L-dopa-induced dyskinesias in drug-naïve parkinsonian monkeys. *Neurobiol.Dis.* 15(2), 171-176.
- HALLETT, P. J. & STANDAERT, D. G. (2004). Rationale for and use of NMDA receptor antagonists in Parkinson's disease. *Pharmacol.Ther.* 102(2), 155-174.
- HALLIDAY, G. M., OPHOF, A., BROE, M., JENSEN, P. H., KETTLE, E., FEDOROW, H., CARTWRIGHT, M. I., GRIFFITHS, F. M., SHEPHERD, C. E., & DOUBLE, K. L. (2005). Alpha-synuclein redistributes to neuromelanin lipid in the substantia nigra early in Parkinson's disease. *Brain* 128(Pt 11), 2654-2664.
- HAMANI, C., RICHTER, E., SCHWALB, J. M., & LOZANO, A. M. (2005). Bilateral subthalamic nucleus stimulation for Parkinson's disease: a systematic review of the clinical literature. *Neurosurgery* 56(6), 1313-1321.
- HAMANI, C., SAINT-CYR, J. A., FRASER, J., KAPLITT, M., & LOZANO, A. M. (2004). The subthalamic nucleus in the context of movement disorders. *Brain* 127(Pt 1), 4-20.
- HASSANI, O. K., FRANCOIS, C., YELNIK, J., & FEGER, J. (1997). Evidence for a dopaminergic innervation of the subthalamic nucleus in the rat. *Brain Res.* 749(1), 88-94.
- HASSANI, O. K., MOUROUX, M., & FEGER, J. (1996). Increased subthalamic neuronal activity after nigral dopaminergic lesion independent of disinhibition via the globus pallidus. *Neuroscience* 72(1), 105-115.
- HAUSER, R. A., HUBBLE, J. P., & TRUONG, D. D. (2003). Randomized trial of the adenosine A(2A) receptor antagonist istradefylline in advanced PD. *Neurology* 61(3), 297-303.
- HAVLICKOVA, M., BLAHOS, J., BRABET, I., LIU, J., HRUSKOVA, B., PREZEAU, L., & PIN, J. P. (2003). The second intracellular loop of metabotropic

glutamate receptors recognizes C termini of G-protein alpha-subunits. *J.Biol.Chem.* 278(37), 35063-35070.

HEALY, D. G., BOU-SLEIMAN, P. M., GIBSON, J. M., ROSS, O. A., JAIN, S., GANDHI, S., GOSAL, D., MUQIT, M. M., WOOD, N. W., & LYNCH, T. (2004). PINK1 (PARK6) associated Parkinson disease in Ireland. *Neurology* 63(8), 1486-1488.

HEDREEN, J. C. (1999). Tyrosine hydroxylase-immunoreactive elements in the human globus pallidus and subthalamic nucleus. *J.Comp Neurol.* 409(3), 400-410.

HEERINGA, M., ABERCROMBIE, E. (1995). Biochemistry of somatodendritic dopamine release in substantia nigra: an *in vivo* comparison with striatal dopamine release. *J. Neurochem.* 65, 192-200

HEFFNER, T. G., HARTMAN, J. A., & SEIDEN, L. S. (1980). A rapid method for the regional dissection of the rat brain. *Pharmacol.Biochem.Behav.* 13(3), 453-456.

HEFTI, F., MELAMED, E., SAHAKIAN, B. J., & WURTMAN, R. J. (1980). Circling behavior in rats with partial, unilateral nigro-striatal lesions: effect of amphetamine, apomorphine, and DOPA. *Pharmacol.Biochem.Behav.* 12(2), 185-188.

HEMMINGS, C., BROOMFIELD, A., BEAN, E., WHITEHEAD, M., & YIP, D. (2009). Immunohistochemical expression of EGFR in colorectal carcinoma correlates with high but not low level gene amplification, as demonstrated by CISH. *Pathology* 41(4), 356-360.

HENDERSON, J. M., CARPENTER, K., CARTWRIGHT, H., & HALLIDAY, G. M. (2000). Loss of thalamic intralaminar nuclei in progressive supranuclear palsy and Parkinson's disease: clinical and therapeutic implications. *Brain* 123 ( Pt 7), 1410-1421.

HERNANDEZ, D., PAISAN, RUIZ C., CRAWLEY, A., MALKANI, R., WERNER, J., GWINN-HARDY, K., DICKSON, D., WAVRANT, DEVRIEZE F., HARDY, J., & SINGLETON, A. (2005a). The dardarin G 2019 S mutation is a common cause of Parkinson's disease but not other neurodegenerative diseases. *Neurosci.Lett.* 389(3), 137-139.

HERNANDEZ, D. G., PAISAN-RUIZ, C., INERNEY-LEO, A., JAIN, S., MEYER-LINDENBERG, A., EVANS, E. W., BERMAN, K. F., JOHNSON, J., AUBURGER, G., SCHAFFER, A. A., LOPEZ, G. J., NUSSBAUM, R. L., & SINGLETON, A. B. (2005b). Clinical and positron emission tomography of Parkinson's disease caused by LRRK2. *Ann.Neurol.* 57(3), 453-456.

HERRERO, M. T., LEVY, R., RUBERG, M., LUQUIN, M. R., VILLARES, J., GUILLEN, J., FAUCHEUX, B., JAVOY-AGID, F., GURIDI, J., AGID, Y., OBESO, J. A., & HIRSCH, E. C. (1996). Consequence of nigrostriatal denervation and L-dopa therapy on the expression of glutamic acid decarboxylase messenger RNA in the pallidum. *Neurology* 47(1), 219-224.

- HESTRIN, S. (1992). Activation and desensitization of glutamate-activated channels mediating fast excitatory synaptic currents in the visual cortex. *Neuron* 9(5), 991-999.
- HILTON, G. D., NUNEZ, J. L., BAMBRICK, L., THOMPSON, S. M., & MCCARTHY, M. M. (2006). Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca<sup>++</sup> from intracellular stores and is prevented by estradiol. *Eur.J.Neurosci.* 24(11), 3008-3016.
- HIRBEC, H., PERESTENKO, O., NISHIMUNE, A., MEYER, G., NAKANISHI, S., HENLEY, J. M., & DEV, K. K. (2002). The PDZ proteins PICK1, GRIP, and syntrophin bind multiple glutamate receptor subtypes. Analysis of PDZ binding motifs. *J.Biol.Chem.* 277(18), 15221-15224.
- HOLLMANN, M., HARTLEY, M., & HEINEMANN, S. (1991). Ca<sup>2+</sup> permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 252(5007), 851-853.
- HOLLMANN, M. & HEINEMANN, S. (1994). Cloned glutamate receptors. *Annu.Rev.Neurosci.* 17, 31-108.
- HORGER, B. A., NISHIMURA, M. C., ARMANINI, M. P., WANG, L. C., POULSEN, K. T., ROSENBLAD, C., KIRIK, D., MOFFAT, B., SIMMONS, L., JOHNSON E JR, MILBRANDT, J., ROSENTHAL, A., BJORKLUND, A., VANDLEN, R. A., HYNES, M. A., & PHILLIPS, H. S. (1998). Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. *J.Neurosci.* 18(13), 4929-4937.
- HORNYKIEWICZ, O. (1966). Dopamine (3-hydroxytyramine) and brain function. *Pharmacol. Rev.* 18,935-964.
- HOWELLS, D. W., PORRITT, M. J., WONG, J. Y., BATCHELOR, P. E., KALNINS, R., HUGHES, A. J., & DONNAN, G. A. (2000). Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. *Exp.Neurol.* 166(1), 127-135.
- HUDTLOFF, C. & THOMSEN, C. (1998). Autoradiographic visualization of group III metabotropic glutamate receptors using [3H]-L-2-amino-4-phosphonobutyrate. *Br.J.Pharmacol.* 124(5), 971-977.
- HUNOT, S., DUGAS, N., FAUCHEUX, B., HARTMANN, A., TARDIEU, M., DEBRE, P., AGID, Y., DUGAS, B., & HIRSCH, E. C. (1999). FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J.Neurosci.* 19(9), 3440-3447.
- HUNTER, R. L., DRAGICEVIC, N., SEIFERT, K., CHOI, D. Y., LIU, M., KIM, H. C., CASS, W. A., SULLIVAN, P. G., & BING, G. (2007). Inflammation induces mitochondrial dysfunction and dopaminergic neurodegeneration in the nigrostriatal system. *J.Neurochem.* 100(5), 1375-1386.
- HUOT, P., LEVESQUE, M., MORISSETTE, M., CALON, F., DRIDI, M., DI, PAOLO T., & PARENT, A. (2008). L-Dopa treatment abolishes the numerical

increase in striatal dopaminergic neurons in parkinsonian monkeys. *J.Chem.Neuroanat.* 35(1), 77-84.

HURLEY, M. J. & JENNER, P. (2006). What has been learnt from study of dopamine receptors in Parkinson's disease? *Pharmacol.Ther.* 111(3), 715-728.

HUTCHISON, W. D., ALLAN, R. J., OPITZ, H., LEVY, R., DOSTROVSKY, J. O., LANG, A. E., & LOZANO, A. M. (1998). Neurophysiological identification of the subthalamic nucleus in surgery for Parkinson's disease. *Ann.Neurol.* 44(4), 622-628.

HUTCHISON, W.D., LOZANO, A.M., DAVIS, K.D., SAINT-CYR, J.A., LANG, A.E., DOSTROVSKY, J.O. (1994). Differential neuronal activity in segments of globus pallidus in Parkinson's disease patients. *Neuroreport.* 5, 1533-1537.

IACOVELLI, L., BRUNO, V., SALVATORE, L., MELCHIORRI, D., GRADINI, R., CARICASOLE, A., BARLETTA, E., DE, BLASI A., & NICOLETTI, F. (2002). Native group-III metabotropic glutamate receptors are coupled to the mitogen-activated protein kinase/phosphatidylinositol-3-kinase pathways. *J.Neurochem.* 82(2), 216-223.

IACOVELLI, L., CAPOBIANCO, L., IULA, M., DI, GIORGI GEREVINI, V., PICASCIA, A., BLAHOS, J., MELCHIORRI, D., NICOLETTI, F., & DE, BLASI A. (2004). Regulation of mGlu4 metabotropic glutamate receptor signaling by type-2 G-protein coupled receptor kinase (GRK2). *Mol.Pharmacol.* 65(5), 1103-1110.

ICZKIEWICZ, J., BROOM, L., COOPER, J., WONG, A., ROSE, S., JENNER, P. (2010). The RGD-containing peptide fragment of osteopontin protects tyrosine hydroxylase positive cells against toxic insult in primary ventral mesencephalic cultures and in the rat substantia nigra. *J. Neurochem.* 114, 1792-1804.

INDEN, M., KITAMURA, Y., TAMAKI, A., YANAGIDA, T., SHIBAIKE, T., YAMAMOTO, A., TAKATA, K., YASUI, H., TAIRA, T., ARIGA, H., TANIGUCHI, T. (2009). Neuroprotective effect of the antiparkinsonian drug pramipexole against nigrostriatal dopaminergic degeneration in rotenone-treated mice. *Neurochem. Int.* 55, 760-767.

IRIBE, Y., MOORE, K., PANG, K. C., & TEPPER, J. M. (1999). Subthalamic stimulation-induced synaptic responses in substantia nigra pars compacta dopaminergic neurons in vitro. *J.Neurophysiol.* 82(2), 925-933.

IVERSEN, L., MULVIHILL, E., HALDEMAN, B., DIEMER, N. H., KAISER, F., SHEARDOWN, M., & KRISTENSEN, P. (1994). Changes in metabotropic glutamate receptor mRNA levels following global ischemia: increase of a putative presynaptic subtype (mGluR4) in highly vulnerable rat brain areas. *J.Neurochem.* 63(2), 625-633.

JACKSON, M. J., SMITH, L. A., AL-BARGHOUTHY, G., ROSE, S., & JENNER, P. (2007). Decreased expression of l-dopa-induced dyskinesia by switching to ropinirole in MPTP-treated common marmosets. *Exp.Neurol.* 204(1), 162-170.

JANKOVIC, J. (2002). Levodopa strengths and weaknesses. *Neurology* 58(4 Suppl 1), S19-S32.

JAVITCH, J. A., UHL, G. R., & SNYDER, S. H. (1984). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: characterization and localization of receptor binding sites in rat and human brain. *Proc.Natl.Acad.Sci.U.S.A* 81(14), 4591-4595.

JAYAKAR, S. S. & DIKSHIT, M. (2004). AMPA receptor regulation mechanisms: future target for safer neuroprotective drugs. *Int.J.Neurosci.* 114(6), 695-734.

JELLINGER, K.A. (1991). Pathology of Parkinson's disease. Changes other than the nigrostriatal pathway. *Mol. Chem. Neuropathol.* 14, 153-197.

JELLINGER, K. A. (1999). Post mortem studies in Parkinson's disease--is it possible to detect brain areas for specific symptoms? *J.Neural Transm.Suppl* 56, 1-29.

JENNER, P. (1989) Clues to the mechanism underlying dopamine cell death in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry.* 52(Suppl) 22-28.

JENNER, P. (2000). Pathophysiology and biochemistry of dyskinesia: clues for the development of non-dopaminergic treatments. *J.Neurol.* 247 Suppl 2, II43-II50.

JENNER, P. (2002). Pharmacology of dopamine agonists in the treatment of Parkinson's disease. *Neurology* 58(4 Suppl 1), S1-S8.

JENNER, P. (2005). A novel dopamine agonist for the transdermal treatment of Parkinson's disease. *Neurology* 65(2 Suppl 1), S3-S5.

JIANG, Q., YAN, Z., & FENG, J. (2006). Activation of group III metabotropic glutamate receptors attenuates rotenone toxicity on dopaminergic neurons through a microtubule-dependent mechanism. *J.Neurosci.* 26(16), 4318-4328.

JIN, X. T. & SMITH, Y. (2007). Activation of presynaptic kainate receptors suppresses GABAergic synaptic transmission in the rat globus pallidus. *Neuroscience* 149(2), 338-349.

JINGAMI, H., NAKANISHI, S., & MORIKAWA, K. (2003). Structure of the metabotropic glutamate receptor. *Curr.Opin.Neurobiol.* 13(3), 271-278.

JOEL, D. & WEINER, I. (1997). The connections of the primate subthalamic nucleus: indirect pathways and the open-interconnected scheme of basal ganglia-thalamocortical circuitry. *Brain Res.Brain Res.Rev.* 23(1-2), 62-78.

JOEL, D. & WEINER, I. (2000). The connections of the dopaminergic system with the striatum in rats and primates: an analysis with respect to the functional and compartmental organization of the striatum. *Neuroscience* 96(3), 451-474.

JOHNSON, K. A., CONN, P. J., & NISWENDER, C. M. (2009). Glutamate receptors as therapeutic targets for Parkinson's disease. *CNS.Neurol.Disord.Drug Targets.* 8(6), 475-491.

KANE-JACKSON, R. & SMITH, Y. (2003). Pre-synaptic kainate receptors in GABAergic and glutamatergic axon terminals in the monkey globus pallidus. *Neuroscience* 122(2), 285-289.

KANG, Y. & KITAI, S. T. (1990). Electrophysiological properties of pedunculopontine neurons and their postsynaptic responses following stimulation of substantia nigra reticulata. *Brain Res.* 535(1), 79-95.

KARUNAKARAN, S., SAEED, U., MISHRA, M., VALLI, R.K., JOSHI, S.D., MEKA, D.P. (2008). Selective activation of p38 mitogen-activated protein kinase in dopaminergic neurons of substantia nigra leads to nuclear translocation of p53 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice. *J. Neurosci.* 28, 12500-12509.

KATAYAMA, J., AKAIKE, N., & NABEKURA, J. (2003). Characterization of pre- and post-synaptic metabotropic glutamate receptor-mediated inhibitory responses in substantia nigra dopamine neurons. *Neurosci.Res.* 45(1), 101-115.

KATZENSCHLAGER, R., SAMPAIO, C., COSTA, J., & LEES, A. (2003). Anticholinergics for symptomatic management of Parkinson's disease. *Cochrane.Database.Syst.Rev.* (2), CD003735.

KAWAGUCHI, Y., WILSON, C. J., AUGOOD, S. J., & EMSON, P. C. (1995). Striatal interneurons: chemical, physiological and morphological characterization. *Trends Neurosci.* 18(12), 527-535.

KEW, J. N. (2004). Positive and negative allosteric modulation of metabotropic glutamate receptors: emerging therapeutic potential. *Pharmacol.Ther.* 104(3), 233-244.

KEW, J. N. & KEMP, J. A. (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)* 179(1), 4-29.

KINCAID, A. E., ZHENG, T., & WILSON, C. J. (1998). Connectivity and convergence of single corticostriatal axons. *J.Neurosci.* 18(12), 4722-4731.

KINOSHITA, A., OHISHI, H., NEKI, A., NOMURA, S., SHIGEMOTO, R., TAKADA, M., NAKANISHI, S., & MIZUNO, N. (1996). Presynaptic localization of a metabotropic glutamate receptor, mGluR8, in the rhinencephalic areas: a light and electron microscope study in the rat. *Neurosci.Lett.* 207(1), 61-64.

KIRIK, D., ROSENBLAD, C., & BJORKLUND, A. (1998). Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp.Neurol.* 152(2), 259-277.

KITA, H. (1993). GABAergic circuits of the striatum. *Prog.Brain Res.* 99, 51-72.

KITA, H. & KITAI, S. T. (1987). Efferent projections of the subthalamic nucleus in the rat: light and electron microscopic analysis with the PHA-L method. *J.Comp Neurol.* 260(3), 435-452.



KITADA, T., ASAKAWA, S., HATTORI, N., MATSUMINE, H., YAMAMURA, Y., MINOSHIMA, S., YOKOCHI, M., MIZUNO, Y., & SHIMIZU, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392(6676), 605-608.

KLEINER-FISMAN, G., HERZOG, J., FISMAN, D. N., TAMMA, F., LYONS, K. E., PAHWA, R., LANG, A. E., & DEUSCHL, G. (2006). Subthalamic nucleus deep brain stimulation: summary and meta-analysis of outcomes. *Mov Disord.* 21 Suppl 14, S290-S304.

KLOCKGETHER, T., TURSKI, L., HONORE, T., ZHANG, Z. M., GASH, D. M., KURLAN, R., & GREENAMYRE, J. T. (1991). The AMPA receptor antagonist NBQX has antiparkinsonian effects in monoamine-depleted rats and MPTP-treated monkeys. *Ann.Neurol.* 30(5), 717-723.

KOLOMIETS, B. P., DENIAU, J. M., GLOWINSKI, J., & THIERRY, A. M. (2003). Basal ganglia and processing of cortical information: functional interactions between trans-striatal and trans-subthalamic circuits in the substantia nigra pars reticulata. *Neuroscience* 117(4), 931-938.

KORDOWER, J. H., EMBORG, M. E., BLOCH, J., MA, S. Y., CHU, Y., LEVENTHAL, L., MCBRIDE, J., CHEN, E. Y., PALFI, S., ROITBERG, B. Z., BROWN, W. D., HOLDEN, J. E., PYZALSKI, R., TAYLOR, M. D., CARVEY, P., LING, Z., TRONO, D., HANTRAYE, P., DEGLON, N., & AEBISCHER, P. (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 290(5492), 767-773.

KORDOWER, J. H., HERZOG, C. D., DASS, B., BAKAY, R. A., STANSELL, J., III, GASMI, M., & BARTUS, R. T. (2006). Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. *Ann.Neurol.* 60(6), 706-715.

KOSINSKI, C. M., RISSO, BRADLEY S., CONN, P. J., LEVEY, A. I., LANDWEHRMEYER, G. B., PENNEY, J. B., JR., YOUNG, A. B., & STANDAERT, D. G. (1999). Localization of metabotropic glutamate receptor 7 mRNA and mGluR7a protein in the rat basal ganglia. *J.Comp Neurol.* 415(2), 266-284.

KRACK, P., POEPPING, M., WEINERT, D., SCHRADER, B., & DEUSCHL, G. (2000). Thalamic, pallidal, or subthalamic surgery for Parkinson's disease? *J.Neurol.* 247 Suppl 2:II122-34., II122-II134.

KRUGER, R., KUHN, W., MULLER, T., WOITALLA, D., GRAEBER, M., KOSEL, S., PRZUNTEK, H., EPPLEN, J. T., SCHOLS, L., & RIESS, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat.Genet.* 18(2), 106-108.

KULKARNI, S., BISHNOI, M., CHOPRA, K. (2009). In vivo microdialysis studies of striatal level of neurotransmitters after haloperidol and chlorpromazine administration. *Indian. J. Exp. Biol.* 47, 91-97

KURYATOV, A., LAUBE, B., BETZ, H., & KUHSE, J. (1994). Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron* 12(6), 1291-1300.

LAFON-CAZAL, M., FAGNI, L., GUIRAUD, M. J., MARY, S., LERNER-NATOLI, M., PIN, J. P., SHIGEMOTO, R., & BOCKAERT, J. (1999a). mGluR7-like metabotropic glutamate receptors inhibit NMDA-mediated excitotoxicity in cultured mouse cerebellar granule neurons. *Eur.J.Neurosci.* 11(2), 663-672.

LAFON-CAZAL, M., VIENNOIS, G., KUHN, R., MALITSCHKE, B., PIN, J. P., SHIGEMOTO, R., & BOCKAERT, J. (1999b). mGluR7-like receptor and GABA(B) receptor activation enhance neurotoxic effects of N-methyl-D-aspartate in cultured mouse striatal GABAergic neurones. *Neuropharmacology.* 38(10), 1631-1640.

LANG, A. E., GILL, S., PATEL, N. K., LOZANO, A., NUTT, J. G., PENN, R., BROOKS, D. J., HOTTON, G., MORO, E., HEYWOOD, P., BRODSKY, M. A., BURCHIEL, K., KELLY, P., DALVI, A., SCOTT, B., STACY, M., TURNER, D., WOOTEN, V. G., ELIAS, W. J., LAWS, E. R., DHAWAN, V., STOESSL, A. J., MATCHAM, J., COFFEY, R. J., & TRAUB, M. (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann.Neurol.* 59(3), 459-466.

LANG, A. E. & LOZANO, A. (1996). Hemiballism in Parkinson's disease. *J.Neurol.Neurosurg.Psychiatry* 60(2), 247.

LANGE, K. W., KORNHUBER, J., & RIEDERER, P. (1997). Dopamine/glutamate interactions in Parkinson's disease. *Neurosci.Biobehav.Rev.* 21(4), 393-400.

LANGSTON, J. W., BALLARD, P., TETRUD, J. W., & IRWIN, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 219(4587), 979-980.

LANGSTON, J. W., FORNO, L. S., TETRUD, J., REEVES, A. G., KAPLAN, J. A., & KARLUK, D. (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann.Neurol.* 46(4), 598-605.

LAVOIE, B. & PARENT, A. (1990). Immunohistochemical study of the serotonergic innervation of the basal ganglia in the squirrel monkey. *J.Comp Neurol.* 299(1), 1-16.

LEE, J. M., ZIPFEL, G. J., & CHOI, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature* 399(6738 Suppl), A7-14.

LERMA, J. (2006). Kainate receptor physiology. *Curr.Opin.Pharmacol.* 6(1), 89-97.

LEROI, I., BRANDT, J., REICH, S. G., LYKETSOS, C. G., GRILL, S., THOMPSON, R., & MARSH, L. (2004). Randomized placebo-controlled trial of donepezil in cognitive impairment in Parkinson's disease. *Int.J.Geriatr.Psychiatry* 19(1), 1-8.

LEROY, E., BOYER, R., AUBURGER, G., LEUBE, B., ULM, G., MEZEY, E., HARTA, G., BROWNSTEIN, M. J., JONNALAGADA, S., CHERNOVA, T., DEHEJIA, A., LAVEDAN, C., GASSER, T., STEINBACH, P. J., WILKINSON, K. D., & POLYMEROPOULOS, M. H. (1998). The ubiquitin pathway in Parkinson's disease. *Nature* 395(6701), 451-452.

LEVESQUE, M., BEDARD, A., COSSETTE, M., & PARENT, A. (2003). Novel aspects of the chemical anatomy of the striatum and its efferents projections. *J.Chem.Neuroanat.* 26(4), 271-281.

LEVY, R., HAZRATI, L. N., HERRERO, M. T., VILA, M., HASSANI, O. K., MOUROUX, M., RUBERG, M., ASENSI, H., AGID, Y., FEGER, J., OBESO, J. A., PARENT, A., & HIRSCH, E. C. (1997). Re-evaluation of the functional anatomy of the basal ganglia in normal and Parkinsonian states. *Neuroscience* 76(2), 335-343.

LEVY, R., HUTCHISON, W. D., LOZANO, A. M., & DOSTROVSKY, J. O. (2000). High-frequency synchronization of neuronal activity in the subthalamic nucleus of parkinsonian patients with limb tremor. *J.Neurosci.* 20(20), 7766-7775.

LEWITT, P. A., LYONS, K. E., & PAHWA, R. (2007). Advanced Parkinson disease treated with rotigotine transdermal system: PREFER Study. *Neurology* 68(16), 1262-1267.

LIN, X., PARASIADOU, L., GU, X.L., WANG, L., SHIM, H., SUN, L., XIE, C., LONG, C.X., YANG, W.J., DING, J., CHEN, Z.Z., GALLANT, P.E., TAO-CHENG, J.H., RUDOW, G., TRONCOSO, J.C., LIU, Z., LI, Z., CAI, H. (2009). Leucine-rich repeat kinase-2 regulates progression of neuropathology induced by Parkinson's disease-related mutant alpha-synuclein. *Neuron*. 64, 807-827.

LITVAN, I., CHESSELET, M. F., GASSER, T., DI MONTE, D. A., PARKER, D., JR., HAGG, T., HARDY, J., JENNER, P., MYERS, R. H., PRICE, D., HALLETT, M., LANGSTON, W. J., LANG, A. E., HALLIDAY, G., ROCCA, W., DUYCKAERTS, C., DICKSON, D. W., BEN-SHLOMO, Y., GOETZ, C. G., & MELAMED, E. (2007). The etiopathogenesis of Parkinson disease and suggestions for future research. Part II. *J.Neuropathol.Exp.Neurol.* 66(5), 329-336.

LIU, F., MA, X. H., ULE, J., BIBB, J. A., NISHI, A., DEMAGGIO, A. J., YAN, Z., NAIRN, A. C., & GREENGARD, P. (2001). Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. *Proc.Natl.Acad.Sci.U.S.A.* 98(20), 11062-11068.

LOPEZ, S., TURLE-LORENZO, N., ACHER, F., DE, LEONIBUS E., MELE, A., & AMALRIC, M. (2007). Targeting group III metabotropic glutamate receptors produces complex behavioral effects in rodent models of Parkinson's disease. *J.Neurosci.* 20;27(25), 6701-6711.

LOSCHMANN, P. A., KUNOW, M., & WACHTEL, H. (1992). Synergism of NBQX with dopamine agonists in the 6-OHDA rat model of Parkinson's disease. *J.Neural Transm.Suppl* 38, 55-64.

- LOSCHMANN, P. A., LANGE, K. W., KUNOW, M., RETTIG, K. J., JAHNIG, P., HONORE, T., TURSKI, L., WACHTEL, H., JENNER, P., & MARSDEN, C. D. (1991). Synergism of the AMPA-antagonist NBQX and the NMDA-antagonist CPP with L-dopa in models of Parkinson's disease. *J.Neural Transm.Park Dis.Dement.Sect.* 3(3), 203-213.
- LOVINGER, D. M. & MCCOOL, B. A. (1995). Metabotropic glutamate receptor-mediated presynaptic depression at corticostriatal synapses involves mGluR2 or 3. *J.Neurophysiol.* 73(3), 1076-1083.
- LOZANO, A. M., LANG, A. E., HUTCHISON, W. D., & DOSTROVSKY, J. O. (1998). New developments in understanding the etiology of Parkinson's disease and in its treatment. *Curr.Opin.Neurobiol.* 8(6), 783-790.
- LUCAS, D. R. & NEWHOUSE, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. *AMA.Arch.Ophthalmol.* 58(2), 193-201.
- LUCKING, C. B., DURR, A., BONIFATI, V., VAUGHAN, J., DE, MICHELE G., GASSER, T., HARHANGI, B. S., MECO, G., DENEFLÉ, P., WOOD, N. W., AGID, Y., & BRICE, A. (2000). Association between early-onset Parkinson's disease and mutations in the parkin gene. *N.Engl.J.Med.* 342(21), 1560-1567.
- LYNCH, M. A. (2004). Long-term potentiation and memory. *Physiol Rev.* 84(1), 87-136.
- MACINNES, N., MESSENGER, M. J., & DUTY, S. (2004). Activation of group III metabotropic glutamate receptors in selected regions of the basal ganglia alleviates akinesia in the reserpine-treated rat. *Br.J.Pharmacol.* 141(1), 15-22.
- MAGILL, P. J., BOLAM, J. P., & BEVAN, M. D. (2001). Dopamine regulates the impact of the cerebral cortex on the subthalamic nucleus-globus pallidus network. *Neuroscience* 106(2), 313-330.
- MAGNIN, M., MOREL, A., & JEANMONOD, D. (2000). Single-unit analysis of the pallidum, thalamus and subthalamic nucleus in parkinsonian patients. *Neuroscience* 96(3), 549-564.
- MAHER, P. (2005). The effects of stress and aging on glutathione metabolism. *Ageing Res.Rev.* 4(2), 288-314.
- MANNING-BOG, A. B., MCCORMACK, A. L., LI, J., UVERSKY, V. N., FINK, A. L., & DI MONTE, D. A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J.Biol.Chem.* 277(3), 1641-1644.
- MANZONI, O., MICHEL, J. M., & BOCKAERT, J. (1997). Metabotropic glutamate receptors in the rat nucleus accumbens. *Eur.J.Neurosci.* 9(7), 1514-1523.
- MARIN, C., JIMENEZ, A., BONASTRE, M., CHASE, T. N., & TOLOSA, E. (2000). Non-NMDA receptor-mediated mechanisms are involved in levodopa-induced motor response alterations in Parkinsonian rats. *Synapse* 36(4), 267-274.

MARINO, M. J., WILLIAMS, D. L., JR., O'BRIEN, J. A., VALENTI, O., MCDONALD, T. P., CLEMENTS, M. K., WANG, R., DILELLA, A. G., HESS, J. F., KINNEY, G. G., & CONN, P. J. (2003). Allosteric modulation of group III metabotropic glutamate receptor 4: a potential approach to Parkinson's disease treatment. *Proc.Natl.Acad.Sci.U.S.A* 100(23), 13668-13673.

MARINOVA-MUTAFCHIEVA, L., SADEGHIAN, M., BROOM, L., DAVIS, J. B., MEDHURST, A. D., & DEXTER, D. T. (2009). Relationship between microglial activation and dopaminergic neuronal loss in the substantia nigra: a time course study in a 6-hydroxydopamine model of Parkinson's disease. *J.Neurochem.* 110(3), 966-975.

MARKS, W. J., JR., OSTREM, J. L., VERHAGEN, L., STARR, P. A., LARSON, P. S., BAKAY, R. A., TAYLOR, R., CAHN-WEINER, D. A., STOESSL, A. J., OLANOW, C. W., & BARTUS, R. T. (2008). Safety and tolerability of intraputamin delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *Lancet Neurol.* 7(5), 400-408.

MATARREDONA, E. R., SANTIAGO, M., VENERO, J. L., CANO, J., & MACHADO, A. (2001). Group II metabotropic glutamate receptor activation protects striatal dopaminergic nerve terminals against MPP<sup>+</sup>-induced neurotoxicity along with brain-derived neurotrophic factor induction. *J.Neurochem.* 76(2), 351-360.

MAURICE, N., DENIAU, J. M., GLOWINSKI, J., & THIERRY, A. M. (1998). Relationships between the prefrontal cortex and the basal ganglia in the rat: physiology of the corticosubthalamic circuits. *J.Neurosci.* 18(22), 9539-9546.

MCCONKEY, D. J., HARTZELL, P., DUDDY, S. K., HAKANSSON, H., & ORRENIUS, S. (1988). 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca<sup>2+</sup>-mediated endonuclease activation. *Science* 242(4876), 256-259.

MCCORMACK, A. L., THIRUCHELVAM, M., MANNING-BOG, A. B., THIFFAULT, C., LANGSTON, J. W., CORY-SLECHTA, D. A., & DI MONTE, D. A. (2002). Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol.Dis.* 10(2), 119-127.

MCCRODDEN, J. M., TIPTON, K. F., & SULLIVAN, J. P. (1990). The neurotoxicity of MPTP and the relevance to Parkinson's disease. *Pharmacol.Toxicol.* 67(1), 8-13.

MCNAUGHT, K. S., BJORKLUND, L. M., BELIZAIRE, R., ISACSON, O., JENNER, P., & OLANOW, C. W. (2002a). Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. *Neuroreport* 13(11), 1437-1441.

MCNAUGHT, K. S., JACKSON, T., JNOBAPTISTE, R., KAPUSTIN, A., & OLANOW, C. W. (2006). Proteasomal dysfunction in sporadic Parkinson's disease. *Neurology* 66(10 Suppl 4), S37-S49.

MCNAUGHT, K.S., JENNER, P. (2001). Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci. Lett.* 297, 191-194.

MCNAUGHT, K. S., MYTILINEOU, C., JNOBAPTISTE, R., YABUT, J., SHASHIDHARAN, P., JENNERT, P., & OLANOW, C. W. (2002b). Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. *J.Neurochem.* 81(2), 301-306.

MCNAUGHT, K.S., PERL, D.P., BROWNELL, A.L., OLANOW, C.W. (2004). Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. *Ann. Neurol.* 56, 149-162.

MCNAUGHT, K. S., OLANOW, C. W., HALLIWELL, B., ISACSON, O., & JENNER, P. (2001). Failure of the ubiquitin-proteasome system in Parkinson's disease. *Nat.Rev.Neurosci.* 2(8), 589-594.

MENA, M. A., CASAREJOS, M. J., CARAZO, A., PAINO, C. L., & GARCIA DE, YEBENES J. (1997). Glia protect fetal midbrain dopamine neurons in culture from L-DOPA toxicity through multiple mechanisms. *J.Neural Transm.* 104(4-5), 317-328.

MERAD-BOUDIA, M., NICOLE, A., SANTIARD-BARON, D., SAILLE, C., & CEBALLOS-PICOT, I. (1998). Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochem.Pharmacol.* 56(5), 645-655.

MEREDITH, G.E., TOTTERDELL, S., PETROSKE, E., SANTA CRUZ, K., CALLISON, R.C., LAU, Y.S. (2002). Lysosomal malfunction accompanies  $\alpha$ -synuclein aggregation in a progressive mouse model of Parkinson's disease. *Brain.Research.* 956, 156-165.

MESSENGER, M. J., DAWSON, L. G., & DUTY, S. (2002). Changes in metabotropic glutamate receptor 1-8 gene expression in the rodent basal ganglia motor loop following lesion of the nigrostriatal tract. *Neuropharmacology* 43(2), 261-271.

MICHEL, P. P. & HEFTI, F. (1990). Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J.Neurosci.Res.* 26(4), 428-435.

MITCHELL, I. J., CLARKE, C. E., BOYCE, S., ROBERTSON, R. G., PEGGS, D., SAMBROOK, M. A., & CROSSMAN, A. R. (1989). Neural mechanisms underlying parkinsonian symptoms based upon regional uptake of 2-deoxyglucose in monkeys exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Neuroscience* 32(1), 213-226.

ITSUKAWA, K., YAMAMOTO, R., OFNER, S., NOZULAK, J., PESCOTT, O., LUKIC, S., STOEHR, N., MOMBEBEAU, C., KUHN, R., MCALLISTER, K. H., VAN DER, PUTTEN H., CRYAN, J. F., & FLOR, P. J. (2005). A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. *Proc.Natl.Acad.Sci.U.S.A* 102(51), 18712-18717.

- MITSUMOTO, A. & NAKAGAWA, Y. (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. *Free Radic.Res.* 35(6), 885-893.
- MIWA, H., KUBO, T., SUZUKI, A., NISHI, K., & KONDO, T. (2005). Retrograde dopaminergic neuron degeneration following intrastriatal proteasome inhibition. *Neurosci.Lett.* 380(1-2), 93-98.
- MOGI, M., TOGARI, A., TANAKA, K., OGAWA, N., ICHINOSE, H., & NAGATSU, T. (2000). Increase in level of tumor necrosis factor-alpha in 6-hydroxydopamine-lesioned striatum in rats is suppressed by immunosuppressant FK506. *Neurosci.Lett.* 289(3), 165-168.
- MONTGOMERY, E. B., JR. (1995). Heavy metals and the etiology of Parkinson's disease and other movement disorders. *Toxicology* 97(1-3), 3-9.
- MONYER, H., SPRENGEL, R., SCHOEPPFER, R., HERB, A., HIGUCHI, M., LOMELI, H., BURNASHEV, N., SAKMANN, B., & SEEBURG, P. H. (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256(5060), 1217-1221.
- MOORE, A. E., CICCHETTI, F., HENNEN, J., & ISACSON, O. (2001). Parkinsonian motor deficits are reflected by proportional A9/A10 dopamine neuron degeneration in the rat. *Exp.Neurol.* 172(2), 363-376.
- MORENS, D. M., DAVIS, J. W., GRANDINETTI, A., ROSS, G. W., POPPER, J. S., & WHITE, L. R. (1996). Epidemiologic observations on Parkinson's disease: incidence and mortality in a prospective study of middle-aged men. *Neurology* 46(4), 1044-1050.
- MORRIS, M. E., HUXHAM, F., MCGINLEY, J., DODD, K., & IANSEK, R. (2001). The biomechanics and motor control of gait in Parkinson disease. *Clin.Biomech.(Bristol, Avon.)* 16(6), 459-470.
- MUELLER, J. C., FUCHS, J., HOFER, A., ZIMPRICH, A., LICHTNER, P., ILLIG, T., BERG, D., WULLNER, U., MEITINGER, T., & GASSER, T. (2005). Multiple regions of alpha-synuclein are associated with Parkinson's disease. *Ann.Neurol.* 57(4), 535-541.
- MULLER, A., REICHMANN, H., LIVERMORE, A., & HUMMEL, T. (2002). Olfactory function in idiopathic Parkinson's disease (IPD): results from cross-sectional studies in IPD patients and long-term follow-up of de-novo IPD patients. *J.Neural Transm.* 109(5-6), 805-811.
- MURER, M. G., DZIEWCZAPOLSKI, G., MENALLED, L. B., GARCIA, M. C., AGID, Y., GERSHANIK, O., & RAISMAN-VOZARI, R. (1998). Chronic levodopa is not toxic for remaining dopamine neurons, but instead promotes their recovery, in rats with moderate nigrostriatal lesions. *Ann.Neurol.* 43(5), 561-575.
- MURRAY, T. K., MESSENGER, M. J., WARD, M. A., WOODHOUSE, S., OSBORNE, D. J., DUTY, S., & O'NEILL, M. J. (2002). Evaluation of the mGluR2/3

agonist LY379268 in rodent models of Parkinson's disease. *Pharmacol.Biochem.Behav.* 73(2), 455-466.

NAGATSU, T., MOGI, M., ICHINOSE, H., & TOGARI, A. (2000). Cytokines in Parkinson's disease. *J.Neural Transm.Suppl* (58), 143-151.

NAKAJIMA, Y., YAMAMOTO, T., NAKAYAMA, T., & NAKANISHI, S. (1999). A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7. *J.Biol.Chem.* 274(39), 27573-27577.

NANDI, D., AZIZ, T. Z., GILADI, N., WINTER, J., & STEIN, J. F. (2002). Reversal of akinesia in experimental parkinsonism by GABA antagonist microinjections in the pedunculopontine nucleus. *Brain* 125(Pt 11), 2418-2430.

NASH, J. E., FOX, S. H., HENRY, B., HILL, M. P., PEGGS, D., MCGUIRE, S., MANEUF, Y., HILLE, C., BROTCHE, J. M., & CROSSMAN, A. R. (2000). Antiparkinsonian actions of ifenprodil in the MPTP-lesioned marmoset model of Parkinson's disease. *Exp.Neurol.* 165(1), 136-142.

NASH, J. E., HILL, M. P., & BROTCHE, J. M. (1999). Antiparkinsonian actions of blockade of NR2B-containing NMDA receptors in the reserpine-treated rat. *Exp.Neurol.* 155(1), 42-48.

NASH, J. E., RAVENSCROFT, P., MCGUIRE, S., CROSSMAN, A. R., MENNITI, F. S., & BROTCHE, J. M. (2004). The NR2B-selective NMDA receptor antagonist CP-101,606 exacerbates L-DOPA-induced dyskinesia and provides mild potentiation of anti-parkinsonian effects of L-DOPA in the MPTP-lesioned marmoset model of Parkinson's disease. *Exp.Neurol.* 188(2), 471-479.

NEUSTADT, B., LIU, H., HAO, J., GREENLEE, W., STAMFORD, A., FOSTER, C., ARIK, L., LACHOWICZ, J., ZHANG, H., BERTORELLI, R., FREDDUZZI, S., VARTY, G., COHEN-WILLIAMS, M., NG, K. (2009). Potent and selective adenosine A<sub>2A</sub> receptor antagonists: 1,2,4-Triazolo[1,5-c]pyrimidines. *Bioorg. Med. Chem. Lett.* 19, 967-971

NICHOLLS, D. & ATTWELL, D. (1990). The release and uptake of excitatory amino acids. *Trends Pharmacol.Sci.* 11(11), 462-468.

NICKLAS, W. J., YOUNGSTER, S. K., KINDT, M. V., & HEIKKILA, R. E. (1987). MPTP, MPP+ and mitochondrial function. *Life Sci.* 40(8), 721-729.

NICOLETTI, F., BRUNO, V., COPANI, A., CASABONA, G., & KNOPFEL, T. (1996). Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci.* 19(7), 267-271.

NIS-OLIVEIRA, R. J., REMIAO, F., CARMO, H., DUARTE, J. A., NAVARRO, A. S., BASTOS, M. L., & CARVALHO, F. (2006). Paraquat exposure as an etiological factor of Parkinson's disease. *Neurotoxicology* 27(6), 1110-1122.

NISWENDER, C. M., JOHNSON, K. A., MILLER, N. R., AYALA, J. E., LUO, Q., WILLIAMS, R., SALEH, S., ORTON, D., WEAVER, C. D., & CONN, P. J. (2010).



Context-dependent pharmacology exhibited by negative allosteric modulators of metabotropic glutamate receptor 7. *Mol.Pharmacol.* 77(3), 459-468.

NISWENDER, C. M., JOHNSON, K. A., WEAVER, C. D., JONES, C. K., XIANG, Z., LUO, Q., RODRIGUEZ, A. L., MARLO, J. E., DE, PAULIS T., THOMPSON, A. D., DAYS, E. L., NALYWAJKO, T., AUSTIN, C. A., WILLIAMS, M. B., AYALA, J. E., WILLIAMS, R., LINDSLEY, C. W., & CONN, P. J. (2008). Discovery, characterization, and antiparkinsonian effect of novel positive allosteric modulators of metabotropic glutamate receptor 4. *Mol.Pharmacol.* 74(5), 1345-1358.

NISWENDER, C. M., LEBOIS, E. P., LUO, Q., KIM, K., MUCHALSKI, H., YIN, H., CONN, P. J., & LINDSLEY, C. W. (2008b). Positive allosteric modulators of the metabotropic glutamate receptor subtype 4 (mGluR4): Part I. Discovery of pyrazolo[3,4-d]pyrimidines as novel mGluR4 positive allosteric modulators. *Bioorg.Med.Chem.Lett.* 18(20), 5626-5630.

O'NEILL, M. J., MURRAY, T. K., CLAY, M. P., LINDSTROM, T., YANG, C. R., & NISENBAUM, E. S. (2005). LY503430: pharmacology, pharmacokinetics, and effects in rodent models of Parkinson's disease. *CNS.Drug Rev.* 11(1), 77-96.

O'NEILL, M. J., MURRAY, T. K., LAKICS, V., VISANJI, N. P., & DUTY, S. (2002). The role of neuronal nicotinic acetylcholine receptors in acute and chronic neurodegeneration. *Curr.Drug Targets.CNS.Neurol.Disord.* 1(4), 399-411.

O'NEILL, M. J., MURRAY, T. K., WHALLEY, K., WARD, M. A., HICKS, C. A., WOODHOUSE, S., OSBORNE, D. J., & SKOLNICK, P. (2004). Neurotrophic actions of the novel AMPA receptor potentiator, LY404187, in rodent models of Parkinson's disease. *Eur.J.Pharmacol.* 486(2), 163-174.

OBESO, J. A., RODRIGUEZ-OROZ, M. C., ITEZ-TEMINO, B., BLESAS, F. J., GURIDI, J., MARIN, C., & RODRIGUEZ, M. (2008). Functional organization of the basal ganglia: therapeutic implications for Parkinson's disease. *Mov Disord.* 23 Suppl 3, S548-S559.

OBESO, J. A., RODRIGUEZ-OROZ, M. C., RODRIGUEZ, M., LANCIEGO, J. L., ARTIEDA, J., GONZALO, N., & OLANOW, C. W. (2000). Pathophysiology of the basal ganglia in Parkinson's disease. *Trends Neurosci.* 23(10 Suppl), S8-19.

OHISHI, H., NEKI, A., & MIZUNO, N. (1998). Distribution of a metabotropic glutamate receptor, mGluR2, in the central nervous system of the rat and mouse: an immunohistochemical study with a monoclonal antibody. *Neurosci.Res.* 30(1), 65-82.

OKAMOTO, N., HORI, S., AKAZAWA, C., HAYASHI, Y., SHIGEMOTO, R., MIZUNO, N., & NAKANISHI, S. (1994). Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. *J.Biol.Chem.* 269(2), 1231-1236.

OLANOW, C. W., AGID, Y., MIZUNO, Y., ALBANESE, A., BONUCCELLI, U., DAMIER, P., DE, YEBENES J., GERSHANIK, O., GUTTMAN, M., GRANDAS, F., HALLETT, M., HORNYKIEWICZ, O., JENNER, P., KATZENSCHLAGER, R., LANGSTON, W. J., LEWITT, P., MELAMED, E., MENA, M. A., MICHEL, P. P.,

MYTILINEOU, C., OBESO, J. A., POEWE, W., QUINN, N., RAISMAN-VOZARI, R., RAJPUT, A. H., RASCOL, O., SAMPAIO, C., & STOCCHI, F. (2004). Levodopa in the treatment of Parkinson's disease: current controversies. *Mov Disord.* 19(9), 997-1005.

OLANOW, C. W. & ARENDASH, G. W. (1994). Metals and free radicals in neurodegeneration. *Curr.Opin.Neurol.* 7(6), 548-558.

OLSSON, M., NIKKHAH, G., BENTLAGE, C., & BJORKLUND, A. (1995). Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *J.Neurosci.* 15(5 Pt 2), 3863-3875.

ORIEUX, G., FRANCOIS, C., FEGER, J., YELNIK, J., VILA, M., RUBERG, M., AGID, Y., & HIRSCH, E. C. (2000). Metabolic activity of excitatory parafascicular and pedunculopontine inputs to the subthalamic nucleus in a rat model of Parkinson's disease. *Neuroscience* 97(1), 79-88.

OUTEIRO, T. F. & LINDQUIST, S. (2003). Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science* 302(5651), 1772-1775.

PACKER, M. A., PORTEOUS, C. M., & MURPHY, M. P. (1996). Superoxide production by mitochondria in the presence of nitric oxide forms peroxynitrite. *Biochem.Mol.Biol.Int.* 40(3), 527-534.

PAHAPILL, P. A. & LOZANO, A. M. (2000). The pedunculopontine nucleus and Parkinson's disease. *Brain* 123 ( Pt 9), 1767-1783.

PAISAN-RUIZ, C., JAIN, S., EVANS, E. W., GILKS, W. P., SIMON, J., VAN DER BRUG M., LOPEZ DE MUNAIN A., APARICIO, S., GIL, A. M., KHAN, N., JOHNSON, J., MARTINEZ, J. R., NICHOLL, D., CARRERA, I. M., PENA, A. S., DE SILVA R., LEES, A., MARTI-MASSO, J. F., PEREZ-TUR, J., WOOD, N. W., & SINGLETON, A. B. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44(4), 595-600.

PALLONE, J. A. (2007). Introduction to Parkinson's disease. *Dis.Mon.* 53(4), 195-199.

PAN, H. S. & WALTERS, J. R. (1988). Unilateral lesion of the nigrostriatal pathway decreases the firing rate and alters the firing pattern of globus pallidus neurons in the rat. *Synapse* 2(6), 650-656.

PARDO, B., MENA, M. A., FAHN, S., & GARCIA DE YEBENES J. (1993). Ascorbic acid protects against levodopa-induced neurotoxicity on a catecholamine-rich human neuroblastoma cell line. *Mov Disord.* 8(3), 278-284.

PARENT, A. (1990). Extrinsic connections of the basal ganglia. *Trends Neurosci.* 13(7), 254-258.

PARENT, A. & CICCETTI, F. (1998). The current model of basal ganglia organization under scrutiny. *Mov Disord.* 13(2), 199-202.

PARENT, A. & HAZRATI, L. N. (1995). Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop. *Brain Res. Brain Res. Rev.* 20(1), 91-127.

PARENT, A., LEVESQUE, M., & PARENT, M. (2001). A re-evaluation of the current model of the basal ganglia. *Parkinsonism. Relat Disord.* 7(3), 193-198.

PARKINSON, J. (1817). *An essay on the shaking palsy*. London: Sherwood, Neeley & Jones

PATIL, J., ZHANG, L., MARTENYI, F., LOWE, S.L., JACKSON, K.A., ANDREEV, B.V., AVEDISOVA, A.S., BARDENSTEIN, L.M., GUROVICH, I.Y., MOROZOVA, M.A., MOSOLOV, S.N., NEZNANOV, N.G., REZNIK, A.M., SMULEVICH, A.B., TOCHILOV, V.A., JOHNSON, B.G., MONN, J.A., SCHOEPP, D.D. (2007). Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat. Med.* 13, 1102-1107.

PATNEAU, D. K. & MAYER, M. L. (1990). Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.* 10(7), 2385-2399.

PAXINOS, G., Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press.

PECKNOLD, J. C., MCCLURE, D. J., APPELTAUER, L., WRZESINSKI, L., & ALLAN, T. (1982). Treatment of anxiety using fenobam (a nonbenzodiazepine) in a double-blind standard (diazepam) placebo-controlled study. *J. Clin. Psychopharmacol.* 2(2), 129-133.

PELKEY, K. A., LAVEZZARI, G., RACCA, C., ROCHE, K. W., & MCBAIN, C. J. (2005). mGluR7 is a metaplastic switch controlling bidirectional plasticity of feedforward inhibition. *Neuron* 46(1), 89-102.

PEPPE, A., PIERANTOZZI, M., BASSI, A., ALTIBRANDI, M. G., BRUSA, L., STEFANI, A., STANZIONE, P., & MAZZONE, P. (2004). Stimulation of the subthalamic nucleus compared with the globus pallidus internus in patients with Parkinson disease. *J. Neurosurg.* 101(2), 195-200.

PERROY, J., PREZEAU, L., DE, WAARD M., SHIGEMOTO, R., BOCKAERT, J., & FAGNI, L. (2000). Selective blockade of P/Q-type calcium channels by the metabotropic glutamate receptor type 7 involves a phospholipase C pathway in neurons. *J. Neurosci.* 20(21), 7896-7904.

PHILLIPS, J. M., LAM, H. A., ACKERSON, L. C., & MAIDMENT, N. T. (2006). Blockade of mGluR glutamate receptors in the subthalamic nucleus ameliorates motor asymmetry in an animal model of Parkinson's disease. *Eur. J. Neurosci.* 23(1), 151-160.

PIALLAT, B., BENAZZOUZ, A., & BENABID, A. L. (1996). Subthalamic nucleus lesion in rats prevents dopaminergic nigral neuron degeneration after striatal 6-

OHDA injection: behavioural and immunohistochemical studies. *Eur.J.Neurosci.* 8(7), 1408-1414.

PICCONI, B., PISANI, A., CENTONZE, D., BATTAGLIA, G., STORTO, M., NICOLETTI, F., BERNARDI, G., & CALABRESI, P. (2002). Striatal metabotropic glutamate receptor function following experimental parkinsonism and chronic levodopa treatment. *Brain.* 125(Pt 12), 2635-2645.

PISANI, A., BONSI, P., CENTONZE, D., GUBELLINI, P., BERNARDI, G., & CALABRESI, P. (2003). Targeting striatal cholinergic interneurons in Parkinson's disease: focus on metabotropic glutamate receptors. *Neuropharmacology* 45(1), 45-56.

PISANI, A., CALABRESI, P., CENTONZE, D., & BERNARDI, G. (1997). Activation of group III metabotropic glutamate receptors depresses glutamatergic transmission at corticostriatal synapse. *Neuropharmacology.* 36(6), 845-851.

PISANI, A., GUBELLINI, P., BONSI, P., CONQUET, F., PICCONI, B., CENTONZE, D., BERNARDI, G., & CALABRESI, P. (2001). Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-D-aspartate responses in medium spiny striatal neurons. *Neuroscience.* 106(3), 579-587.

POLYMEROPOULOS, M. H., LAVEDAN, C., LEROY, E., IDE, S. E., DEHEJIA, A., DUTRA, A., PIKE, B., ROOT, H., RUBENSTEIN, J., BOYER, R., STENROOS, E. S., CHANDRASEKHARAPPA, S., ATHANASSIADOU, A., PAPAPETROPOULOS, T., JOHNSON, W. G., LAZZARINI, A. M., DUVOISIN, R. C., DI, IORIO G., GOLBE, L. I., & NUSSBAUM, R. L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276(5321), 2045-2047.

PONSEN, M. M., STOFFERS, D., BOOIJ, J., VAN ECK-SMIT, B. L., WOLTERS, E. C., & BERENDSE, H. W. (2004). Idiopathic hyposmia as a preclinical sign of Parkinson's disease. *Ann.Neurol.* 56(2), 173-181.

PORTER, R. H., GREENE, J. G., HIGGINS, D. S., JR., & GREENAMYRE, J. T. (1994). Polysynaptic regulation of glutamate receptors and mitochondrial enzyme activities in the basal ganglia of rats with unilateral dopamine depletion. *J.Neurosci.* 14(11 Pt 2), 7192-7199.

PORTER, R. H., JAESCHKE, G., SPOOREN, W., BALLARD, T. M., BUTTELMANN, B., KOLCZEWSKI, S., PETERS, J. U., PRINSSSEN, E., WICHMANN, J., VIEIRA, E., MUHLEMANN, A., GATTI, S., MUTEL, V., & MALHERBE, P. (2005). Fenobam: a clinically validated nonbenzodiazepine anxiolytic is a potent, selective, and noncompetitive mGlu5 receptor antagonist with inverse agonist activity. *J.Pharmacol.Exp.Ther.* 315(2), 711-721.

PRZEDBORSKI, S., LEVIVIER, M., JIANG, H., FERREIRA, M., JACKSON-LEWIS, V., DONALDSON, D., & TOGASAKI, D. M. (1995). Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience* 67(3), 631-647.

PRZEDBORSKI, S., JACKSON-LEWIS, V., NAINI, A.B., JAKOWEC, M., PETZINGER, G., MILLER, R., AKRAM, M. (2001). The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. *J.Neurochem.* 76, 1265-1274.

RAJPUT, A. H., FENTON, M., BIRDI, S., & MACAULAY, R. (1997). Is levodopa toxic to human substantia nigra? *Mov Disord.* 12(5), 634-638.

RAMSAY, R. R., KRUEGER, M. J., YOUNGSTER, S. K., GLUCK, M. R., CASIDA, J. E., & SINGER, T. P. (1991). Interaction of 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. *J.Neurochem.* 56(4), 1184-1190.

RAO, S. P. & SIKDAR, S. K. (2006). Astrocytes in 17beta-estradiol treated mixed hippocampal cultures show attenuated calcium response to neuronal activity. *Glia* 53(8), 817-826.

RASCOL, O. (2000). The pharmacological therapeutic management of levodopa-induced dyskinesias in patients with Parkinson's disease. *J.Neurol.* 247 Suppl 2, II51-II57.

REN, Y., LIU, W., JIANG, H., JIANG, Q., & FENG, J. (2005). Selective vulnerability of dopaminergic neurons to microtubule depolymerization. *J.Biol.Chem.* 280(40), 34105-34112.

RIDEOUT, H. J., LANG-ROLLIN, I. C., SAVALLE, M., & STEFANIS, L. (2005). Dopaminergic neurons in rat ventral midbrain cultures undergo selective apoptosis and form inclusions, but do not up-regulate iHSP70, following proteasomal inhibition. *J.Neurochem.* 93(5), 1304-1313.

RIDEOUT, H. J., LARSEN, K. E., SULZER, D., & STEFANIS, L. (2001). Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. *J.Neurochem.* 78(4), 899-908.

RINVIK, E. & OTTERSEN, O. P. (1993). Terminals of subthalamonigral fibres are enriched with glutamate-like immunoreactivity: an electron microscopic, immunogold analysis in the cat. *J.Chem.Neuroanat.* 6(1), 19-30.

ROBLEDO, P. & FEGER, J. (1991). Acute monoaminergic depletion in the rat potentiates the excitatory effect of the subthalamic nucleus in the substantia nigra pars reticulata but not in the pallidal complex. *J.Neural Transm.Gen.Sect.* 86(2), 115-126.

RODRIGUEZ, M. C., OBESO, J. A., & OLANOW, C. W. (1998). Subthalamic nucleus-mediated excitotoxicity in Parkinson's disease: a target for neuroprotection. *Ann.Neurol.* 44(3 Suppl 1), S175-S188.

RODRIGUEZ-MORENO, A., HERRERAS, O., & LERMA, J. (1997). Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. *Neuron* 19(4), 893-901.

ROHE, C. F., MONTAGNA, P., BREEDVELD, G., CORTELLI, P., OOSTRA, B. A., & BONIFATI, V. (2004). Homozygous PINK1 C-terminus mutation causing early-onset parkinsonism. *Ann.Neurol.* 56(3), 427-431.

ROSE, S., JACKSON, M. J., SMITH, L. A., STOCKWELL, K., JOHNSON, L., CARMINATI, P., & JENNER, P. (2006). The novel adenosine A2a receptor antagonist ST1535 potentiates the effects of a threshold dose of L-DOPA in MPTP treated common marmosets. *Eur.J.Pharmacol.* 546(1-3), 82-87.

ROSE, S., RAMSAY, CROFT N., & JENNER, P. (2007). The novel adenosine A2a antagonist ST1535 potentiates the effects of a threshold dose of l-dopa in unilaterally 6-OHDA-lesioned rats. *Brain Res.* 1133(1), 110-114.

ROSENMUND, C., STERN-BACH, Y., & STEVENS, C. F. (1998). The tetrameric structure of a glutamate receptor channel. *Science* 280(5369), 1596-1599.

ROSS, G. W., ABBOTT, R. D., PETROVITCH, H., MORENS, D. M., GRANDINETTI, A., TUNG, K. H., TANNER, C. M., MASAKI, K. H., BLANCHETTE, P. L., CURB, J. D., POPPER, J. S., & WHITE, L. R. (2000). Association of coffee and caffeine intake with the risk of Parkinson disease. *JAMA* 283(20), 2674-2679.

ROUSE, S. T., MARINO, M. J., BRADLEY, S. R., AWAD, H., WITTMANN, M., & CONN, P. J. (2000). Distribution and roles of metabotropic glutamate receptors in the basal ganglia motor circuit: implications for treatment of Parkinson's disease and related disorders. *Pharmacol.Ther.* 88(3), 427-435.

SALT, T. E. & TURNER, J. P. (1998). Modulation of sensory inhibition in the ventrobasal thalamus via activation of group II metabotropic glutamate receptors by 2R,4R-aminopyrrolidine-2,4-dicarboxylate. *Exp.Brain Res.* 121(2), 181-185.

SANBERG, P. (1980). Haloperidol-induced catalepsy is mediated by postsynaptic dopamine receptors. *Nature.* 284, 472-473.

SANGHERA, M. K., MANAYE, K., MCMAHON, A., SONSALLA, P. K., & GERMAN, D. C. (1997). Dopamine transporter mRNA levels are high in midbrain neurons vulnerable to MPTP. *Neuroreport* 8(15), 3327-3331.

SAPORITO, M.S., THOMAS, B.A., SCOTT, R.W. (2000). MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons *in vivo*. *J. Neurochem.* 75, 1200-1208.

SARAVANAN, K.S., SINDHU, K.M., SENTHILKUMAR, K.S., MOHANAKUMAR, K.P. (2006). L-deprenyl protects against rotenone-induced, oxidative stress-mediated dopaminergic neurodegeneration in rats. *Neurochem. Int.* 49, 28-40.

SATO, F., PARENT, M., LEVESQUE, M., & PARENT, A. (2000). Axonal branching pattern of neurons of the subthalamic nucleus in primates. *J.Comp Neurol.* 424(1), 142-152.

- SAUGSTAD, J. A., SEGERSON, T. P., & WESTBROOK, G. L. (1996). Metabotropic glutamate receptors activate G-protein-coupled inwardly rectifying potassium channels in *Xenopus* oocytes. *J.Neurosci.* 16(19), 5979-5985.
- SCANZIANI, M., SALIN, P. A., VOGT, K. E., MALENKA, R. C., & NICOLL, R. A. (1997). Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. *Nature.* 385(6617), 630-634.
- SCHAPIRA, A. H., COOPER, J. M., DEXTER, D., JENNER, P., CLARK, J. B., & MARSDEN, C. D. (1989). Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* 1(8649), 1269.
- SCHENCK, C. H. & MAHOWALD, M. W. (2002). REM sleep behavior disorder: clinical, developmental, and neuroscience perspectives 16 years after its formal identification in SLEEP. *Sleep* 25(2), 120-138.
- SCHLOSSMACHER, M. G., FROSCH, M. P., GAI, W. P., MEDINA, M., SHARMA, N., FORNO, L., OCHIISHI, T., SHIMURA, H., SHARON, R., HATTORI, N., LANGSTON, J. W., MIZUNO, Y., HYMAN, B. T., SELKOE, D. J., & KOSIK, K. S. (2002). Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am.J.Pathol.* 160(5), 1655-1667.
- SCHOEPP, D. D. (2001). Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. *J.Pharmacol.Exp.Ther.* 299(1), 12-20.
- SCHOUSBOE, A., SARUP, A., BAK, L. K., WAAGEPETERSEN, H. S., & LARSSON, O. M. (2004). Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. *Neurochem.Int.* 45(4), 521-527.
- SHERER, T. B., BETARBET, R., & GREENAMYRE, J. T. (2002). Environment, mitochondria, and Parkinson's disease. *Neuroscientist.* 8(3), 192-197.
- SHERER, T.B., BETARBET, R., KIM, J.H., GREENAMYRE, J.T. (2003). Selective microglial activation in the rat rotenone model of Parkinson's disease. *Neurosci. Lett.* 341, 87-90.
- SHERER, T. B., RICHARDSON, J. R., TESTA, C. M., SEO, B. B., PANOV, A. V., YAGI, T., MATSUNO-YAGI, A., MILLER, G. W., & GREENAMYRE, J. T. (2007). Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. *J.Neurochem.* 100(6), 1469-1479.
- SHIGEMOTO, R., KINOSHITA, A., WADA, E., NOMURA, S., OHISHI, H., TAKADA, M., FLOR, P. J., NEKI, A., ABE, T., NAKANISHI, S., & MIZUNO, N. (1997). Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J.Neurosci.* 17(19), 7503-7522.
- SHIMOJI, M., ZHANG, L., MANDIR, A.S., DAWSON, V.L., DAWSON, T.M. (2005). Absence of inclusion body formation in the MPTP mouse model of Parkinson's disease. *Brain. Res. Mol. Brain. Res.* 134, 103-108.

SHIOZAKI, S., ICHIKAWA, S., NAKAMURA, J., KITAMURA, S., YAMADA, K., KUWANA. (1999). Actions of adenosine A2A receptor antagonist KW-6002 on drug-induced catalepsy and hypokinesia caused by reserpine or MPTP. *Psychopharm. (Berl)*. 147, 90-95.

SHULTS, C. W., OAKES, D., KIEBURTZ, K., BEAL, M. F., HAAS, R., PLUMB, S., JUNCOS, J. L., NUTT, J., SHOULSON, I., CARTER, J., KOMPOLITI, K., PERLMUTTER, J. S., REICH, S., STERN, M., WATTS, R. L., KURLAN, R., MOLHO, E., HARRISON, M., & LEW, M. (2002). Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. *Arch.Neurol*. 59(10), 1541-1550.

SIMAN, R., NOSZEK, J. C., & KEGERISE, C. (1989). Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage. *J.Neurosci*. 9(5), 1579-1590.

SIMONYI, A., MILLER, L. A., & SUN, G. Y. (2000). Region-specific decline in the expression of metabotropic glutamate receptor 7 mRNA in rat brain during aging. *Brain Res.Mol.Brain Res*. 82(1-2), 101-106.

SINCLAIR, C., REAVY, H., GRIEVE, A., SCHOUSBOE, A., MORELLI, E., NOVELLINO, E., CAMPIANI, G., & GRIFFITHS, R. (2003). Inherent desensitisation-preventing properties of a novel, subtype-selective AMPA receptor agonist, (S)-CPW 399, as a possible explanation for its excitotoxic action in cultured cerebellar granule cells. *Neurochem.Int*. 42(6), 499-510.

SMITH, A. D. & BOLAM, J. P. (1990). The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends Neurosci*. 13(7), 259-265.

SMITH, Y., BEVAN, M. D., SHINK, E., & BOLAM, J. P. (1998). Microcircuitry of the direct and indirect pathways of the basal ganglia. *Neuroscience* 86(2), 353-387.

SMITH, Y., CHARARA, A., HANSON, J. E., PAQUET, M., & LEVEY, A. I. (2000). GABA(B) and group I metabotropic glutamate receptors in the striatopallidal complex in primates. *J.Anat*. 196(Pt 4), 555-576.

SONSALLA, P. K., ALBERS, D. S., & ZEEVALK, G. D. (1998). Role of glutamate in neurodegeneration of dopamine neurons in several animal models of parkinsonism. *Amino.Acids* 14(1-3), 69-74.

SPILLANTINI, M. G., SCHMIDT, M. L., LEE, V. M., TROJANOWSKI, J. Q., JAKES, R., & GOEDERT, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* 388(6645), 839-840.

SPILLANTINI, M.G., CROWTHER, R.A., JAKES, R., HASEGAWA, M., GOEDERT, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl. Acad. Sci. USA*. 95, 6469-6473.



STANFORD, I. M. & COOPER, A. J. (1999). Presynaptic mu and delta opioid receptor modulation of GABAA IPSCs in the rat globus pallidus in vitro. *J.Neurosci.* 19(12), 4796-4803.

STARR, M. S. (1998). Antagonists of glutamate in the treatment of Parkinson's disease: from laboratory to the clinic. *Amino.Acids* 14(1-3), 41-42.

STEECE-COLLIER, K., COLLIER, T. J., SLADEK, C. D., & SLADEK, J. R., JR. (1990) Chronic levodopa impairs morphological development of grafted embryonic dopamine neurons. *Exp.Neurol.* 110(2), 201-208.

SUKOFF-RIZZO, S., LEONARD, SK., GILBERT, A., DOLLINGS, P., SMITH, DL., ZHANG, M-Y. (2010). The mGluR7 allosteric agonist AMN082 is a monoaminergic agent in disguise! *SFN Abstract* Nov 2010: 643.28.

SWANSON, C., BURES, M., JOHNSON, M., LINDEN, A., MONN, J., SCHOEPP, D. (2005). Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. *Nat.Rev.Drug Discovery.* 4(2), 131-144.

TAKECHI, H., EILERS, J., & KONNERTH, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. *Nature* 396(6713), 757-760.

TAN, E. K., YEW, K., CHUA, E., PUVAN, K., SHEN, H., LEE, E., PUONG, K. Y., ZHAO, Y., PAVANNI, R., WONG, M. C., JAMORA, D., DE, SILVA D., MOE, K. T., WOON, F. P., YUEN, Y., & TAN, L. (2006). PINK1 mutations in sporadic early-onset Parkinson's disease. *Mov Disord.* 21(6), 789-793.

TAN, S., WOOD, M., & MAHER, P. (1998). Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J.Neurochem.* 71(1), 95-105.

TANDE, D., HOGLINGER, G., DEBEIR, T., FREUNDLIEB, N., HIRSCH, E. C., & FRANCOIS, C. (2006). New striatal dopamine neurons in MPTP-treated macaques result from a phenotypic shift and not neurogenesis. *Brain* 129(Pt 5), 1194-1200.

TANNER, C. M. (2003). Is the cause of Parkinson's disease environmental or hereditary? Evidence from twin studies. *Adv.Neurol.* 91, 133-142.

TANSEY, M. G. & GOLDBERG, M. S. (2010). Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. *Neurobiol.Dis.* 37(3), 510-518.

TASKER, R. R. (1997). Selection of patients for surgery for Parkinson's disease. *Clin.Neurosurg.* 44, 63-67.

TAYLOR, D. L., DIEMEL, L. T., & POCOCK, J. M. (2003). Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. *J.Neurosci.* 23(6), 2150-2160.

TEPPER, J. M., KOOS, T., & WILSON, C. J. (2004). GABAergic microcircuits in the neostriatum. *Trends Neurosci.* 27(11), 662-669.

TESTA, C. M., FRIBERG, I. K., WEISS, S. W., & STANDAERT, D. G. (1998). Immunohistochemical localization of metabotropic glutamate receptors mGluR1a and mGluR2/3 in the rat basal ganglia. *J.Comp Neurol.* 390(1), 5-19.

TESTA, C. M., STANDAERT, D. G., YOUNG, A. B., & PENNEY, J. B., JR. (1994). Metabotropic glutamate receptor mRNA expression in the basal ganglia of the rat. *J.Neurosci.* 14(5 Pt 2), 3005-3018.

THIRUCHELVAM, M., BROCKEL, B.J., RICHFIELD, E.K., BAGGS, R.B., CORY-SLECHTA, D.A. (2000). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems; environmental risk factors for Parkinson's disease? *Brain. Res.* 873, 225-234.

THOMAS, J., WANG, J., TAKUBO, H., SHENG, J., DE, JESUS S., & BANKIEWICZ, K. S. (1994). A 6-hydroxydopamine-induced selective parkinsonian rat model: further biochemical and behavioral characterization. *Exp.Neurol.* 126(2), 159-167.

THOMAS, N. K., WRIGHT, R. A., HOWSON, P. A., KINGSTON, A. E., SCHOEPP, D. D., & JANE, D. E. (2001). (S)-3,4-DCPG, a potent and selective mGlu8a receptor agonist, activates metabotropic glutamate receptors on primary afferent terminals in the neonatal rat spinal cord. *Neuropharmacology* 40(3), 311-318.

THOMSEN, C. & HAMPSON, D. R. (1999). Contribution of metabotropic glutamate receptor mGluR4 to L-2-[3H]amino-4-phosphonobutyrate binding in mouse brain. *J.Neurochem.* 72(2), 835-840.

TINTNER, R. & JANKOVIC, J. (2002). Treatment options for Parkinson's disease. *Curr.Opin.Neurol.* 15(4), 467-476.

TROMBLEY, P. Q. & WESTBROOK, G. L. (1992). L-AP4 inhibits calcium currents and synaptic transmission via a G-protein-coupled glutamate receptor. *J.Neurosci.* 12(6), 2043-2050.

TSENG, K. Y., RIQUELME, L. A., BELFORTE, J. E., PAZO, J. H., & MURER, M. G. (2000). Substantia nigra pars reticulata units in 6-hydroxydopamine-lesioned rats: responses to striatal D2 dopamine receptor stimulation and subthalamic lesions. *Eur.J.Neurosci.* 12(1), 247-256.

TU, J. C., XIAO, B., NAISBITT, S., YUAN, J. P., PETRALIA, R. S., BRAKEMAN, P., DOAN, A., AAKALU, V. K., LANAHAAN, A. A., SHENG, M., & WORLEY, P. F. (1999). Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* 23(3), 583-592.

TU, J. C., XIAO, B., YUAN, J. P., LANAHAAN, A. A., LEOFFERT, K., LI, M., LINDEN, D. J., & WORLEY, P. F. (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron.* 21(4), 717-726.

TURNER, J. P. & SALT, T. E. (1999). Group III metabotropic glutamate receptors control corticothalamic synaptic transmission in the rat thalamus in vitro. *J.Physiol.* 519 Pt 2:481-91., 481-491.

TURNER, J. P. & SALT, T. E. (2003). Group II and III metabotropic glutamate receptors and the control of the nucleus reticularis thalami input to rat thalamocortical neurones in vitro. *Neuroscience.* 122(2), 459-469.

UGOLINI, A., LARGE, C. H., & CORSI, M. (2008). AMN082, an allosteric mGluR7 agonist that inhibits afferent glutamatergic transmission in rat basolateral amygdala. *Neuropharmacology.* 55(4), 532-536.

UNGERSTEDT, U. (1968). 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. *Eur J Pharmacol.* 5, 107-110.

UNGERSTEDT, U. (1971c). Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol Scand.Suppl* 367, 95-122.

UNGERSTEDT, U. (1971b). Postsynaptic supersensitivity after 6-hydroxy-dopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol Scand.Suppl* 367, 69-93.

UNGERSTEDT, U. (1971a). Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour. *Acta Physiol Scand.Suppl* 367, 49-68.

URLINGER, S., KUCHLER, K., MEYER, T. H., UEBEL, S., & TAMPE, R. (1997). Intracellular location, complex formation, and function of the transporter associated with antigen processing in yeast. *Eur.J.Biochem.* 245(2), 266-272.

VALENTE, E. M., BOU-SLEIMAN, P. M., CAPUTO, V., MUQIT, M. M., HARVEY, K., GISPERT, S., ALI, Z., DEL, TURCO D., BENTIVOGLIO, A. R., HEALY, D. G., ALBANESE, A., NUSSBAUM, R., GONZALEZ-MALDONADO, R., DELLER, T., SALVI, S., CORTELLI, P., GILKS, W. P., LATCHMAN, D. S., HARVEY, R. J., DALLAPICCOLA, B., AUBURGER, G., & WOOD, N. W. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304(5674), 1158-1160.

VALENTI, O., MANNAIONI, G., SEABROOK, G. R., CONN, P. J., & MARINO, M. J. (2005). Group III metabotropic glutamate-receptor-mediated modulation of excitatory transmission in rodent substantia nigra pars compacta dopamine neurons. *J.Pharmacol.Exp.Ther.* 313(3), 1296-1304.

VALENTI, O., MARINO, M. J., WITTMANN, M., LIS, E., DILELLA, A. G., KINNEY, G. G., & CONN, P. J. (2003). Group III metabotropic glutamate receptor-mediated modulation of the striatopallidal synapse. *J.Neurosci.* 23(18), 7218-7226.

VAN DOMBURG, P. H. & TEN DONKELAAR, H. J. (1991). The human substantia nigra and ventral tegmental area. A neuroanatomical study with notes on aging and aging diseases. *Adv.Anat.Embryol.Cell Biol.* 121, 1-132.

VARSHAVSKY, A. (1996). The N-end rule: functions, mysteries, uses. *Proc.Natl.Acad.Sci.U.S.A* 93(22), 12142-12149.

VERHAGEN, METMAN L., DEL, DOTTO P., VAN DEN, MUNCKHOF P., FANG, J., MOURADIAN, M. M., & CHASE, T. N. (1998). Amantadine as treatment for dyskinesias and motor fluctuations in Parkinson's disease. *Neurology* 50(5), 1323-1326.

VERNON, A. C., PALMER, S., DATLA, K. P., ZBARSKY, V., CROUCHER, M. J., & DEXTER, D. T. (2005). Neuroprotective effects of metabotropic glutamate receptor ligands in a 6-hydroxydopamine rodent model of Parkinson's disease. *Eur.J.Neurosci.* 22(7), 1799-1806.

VERNON, A. C., ZBARSKY, V., DATLA, K. P., CROUCHER, M. J., & DEXTER, D. T. (2007a). Subtype selective antagonism of substantia nigra pars compacta Group I metabotropic glutamate receptors protects the nigrostriatal system against 6-hydroxydopamine toxicity in vivo. *J.Neurochem.* 103(3), 1075-1091.

VERNON, A. C., ZBARSKY, V., DATLA, K. P., DEXTER, D. T., & CROUCHER, M. J. (2007b). Selective activation of group III metabotropic glutamate receptors by L-(+)-2-amino-4-phosphonobutyric acid protects the nigrostriatal system against 6-hydroxydopamine toxicity in vivo. *J.Pharmacol.Exp.Ther.* 320(1), 397-409.

VILA, M., LEVY, R., HERRERO, M. T., RUBERG, M., FAUCHEUX, B., OBESO, J. A., AGID, Y., & HIRSCH, E. C. (1997). Consequences of nigrostriatal denervation on the functioning of the basal ganglia in human and nonhuman primates: an in situ hybridization study of cytochrome oxidase subunit I mRNA. *J.Neurosci.* 17(2), 765-773.

VILA, M., MARIN, C., RUBERG, M., JIMENEZ, A., RAISMAN-VOZARI, R., AGID, Y., TOLOSA, E., & HIRSCH, E. C. (1999b). Systemic administration of NMDA and AMPA receptor antagonists reverses the neurochemical changes induced by nigrostriatal denervation in basal ganglia. *J.Neurochem.* 73(1), 344-352.

VISANJI, N. P., O'NEILL, M. J., & DUTY, S. (2006). Nicotine, but neither the alpha4beta2 ligand RJR2403 nor an alpha7 nAChR subtype selective agonist, protects against a partial 6-hydroxydopamine lesion of the rat median forebrain bundle. *Neuropharmacology* 51(3), 506-516. 2006.

VON CAMPENHAUSEN S., BORNSCHEIN, B., WICK, R., BOTZEL, K., SAMPAIO, C., POEWE, W., OERTEL, W., SIEBERT, U., BERGER, K., & DODEL, R. (2005). Prevalence and incidence of Parkinson's disease in Europe. *Eur.Neuropsychopharmacol.* 15(4), 473-490.

WAAGEPETERSEN, H. S., QU, H., SONNEWALD, U., SHIMAMOTO, K., & SCHOUSBOE, A. (2005). Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. *Neurochem.Int.* 47(1-2), 92-102.

WACHTEL, H., KUNOW, M., & LOSCHMANN, P. A. (1992). NBQX (6-nitro-sulfamoyl-benzo-quinoxaline-dione) and CPP (3-carboxy-piperazin-propyl

phosphonic acid) potentiate dopamine agonist induced rotations in substantia nigra lesioned rats. *Neurosci.Lett.* 142(2), 179-182.

WALLACE, B. A., ASHKAN, K., HEISE, C. E., FOOTE, K. D., TORRES, N., MITROFANIS, J., & BENABID, A. L. (2007). Survival of midbrain dopaminergic cells after lesion or deep brain stimulation of the subthalamic nucleus in MPTP-treated monkeys. *Brain* 130(Pt 8), 2129-2145.

WALTER, B. L. & VITEK, J. L. (2004). Surgical treatment for Parkinson's disease. *Lancet Neurol.* 3(12), 719-728.

WANG, H., JIANG, W., YANG, R., & LI, Y. (2011). Spinal metabotropic glutamate receptor 4 is involved in neuropathic pain. *Neuroreport* 22(5), 244-248.

WANG, H. L., CHOU, A. H., YEH, T. H., LI, A. H., CHEN, Y. L., KUO, Y. L., TSAI, S. R., & YU, S. T. (2007a). PINK1 mutants associated with recessive Parkinson's disease are defective in inhibiting mitochondrial release of cytochrome c. *Neurobiol.Dis.* 28(2), 216-226.

WANG, Q., CHANG, L., ROWAN, M. J., & ANWYL, R. (2007b). Developmental dependence, the role of the kinases p38 MAPK and PKC, and the involvement of tumor necrosis factor-R1 in the induction of mGlu-5 LTD in the dentate gyrus. *Neuroscience* 144(1), 110-118.

WANG, X.F., LI, S., CHOU, A.P., BRONSTEIN, J.M. (2007c). Inhibitory effects of pesticides on proteasome activity: implications in Parkinson's disease. *Neurobiol. Dis.* 23, 198-205.

WARAGAI, M., NAKAI, M., WEI, J., FUJITA, M., MIZUNO, H., HO, G., MASLIAH, E., AKATSU, H., YOKOCHI, F., & HASHIMOTO, M. (2007). Plasma levels of DJ-1 as a possible marker for progression of sporadic Parkinson's disease. *Neurosci.Lett.* 425(1), 18-22.

WATTS, R. L., JANKOVIC, J., WATERS, C., RAJPUT, A., BOROOJERDI, B., & RAO, J. (2007). Randomized, blind, controlled trial of transdermal rotigotine in early Parkinson disease. *Neurology* 68(4), 272-276.

WHITTON, P. S. (2007). Inflammation as a causative factor in the aetiology of Parkinson's disease. *Br.J.Pharmacol.* 150(8), 963-976.

WHONE, A. L., WATTS, R. L., STOESSL, A. J., DAVIS, M., RESKE, S., NAHMIAS, C., LANG, A. E., RASCOL, O., RIBEIRO, M. J., REMY, P., POEWE, W. H., HAUSER, R. A., & BROOKS, D. J. (2003). Slower progression of Parkinson's disease with ropinirole versus levodopa: The REAL-PET study. *Ann.Neurol.* 54(1), 93-101.

WIGMORE, M. A. & LACEY, M. G. (1998). Metabotropic glutamate receptors depress glutamate-mediated synaptic input to rat midbrain dopamine neurones in vitro. *Br.J.Pharmacol.* 123(4), 667-674.

- WILLIAMS, R., NISWENDER, C. M., LUO, Q., LE, U., CONN, P. J., & LINDSLEY, C. W. (2009). Positive allosteric modulators of the metabotropic glutamate receptor subtype 4 (mGluR4). Part II: Challenges in hit-to-lead. *Bioorg.Med.Chem.Lett.* 19(3), 962-966.
- WINDELS, F., CARCENAC, C., POUPARD, A., & SAVASTA, M. (2005). Pallidal origin of GABA release within the substantia nigra pars reticulata during high-frequency stimulation of the subthalamic nucleus. *J.Neurosci.* 25(20), 5079-5086.
- WITTMANN, M., MARINO, M. J., BRADLEY, S. R., & CONN, P. J. (2001). Activation of group III mGluRs inhibits GABAergic and glutamatergic transmission in the substantia nigra pars reticulata. *J.Neurophysiol.* 85(5), 1960-1968.
- WITTMANN, M., MARINO, M. J., & CONN, P. J. (2002). Dopamine modulates the function of group II and group III metabotropic glutamate receptors in the substantia nigra pars reticulata. *J.Pharmacol.Exp.Ther.* 302(2), 433-441.
- WOOD, M. R., HOPKINS, C. R., BROGAN, J. T., CONN, P. J., & LINDSLEY, C. W. (2011). "Molecular switches" on mGluR allosteric ligands that modulate modes of pharmacology. *Biochemistry* 50(13), 2403-2410.
- WROBLEWSKA, B., SANTI, M. R., & NEALE, J. H. (1998). N-acetylaspartylglutamate activates cyclic AMP-coupled metabotropic glutamate receptors in cerebellar astrocytes. *Glia.* 24(2), 172-179.
- WU, D. C., JACKSON-LEWIS, V., VILA, M., TIEU, K., TEISMANN, P., VADSETH, C., CHOI, D. K., ISCHIROPOULOS, H., & PRZEDBORSKI, S. (2002). Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J.Neurosci.* 22(5), 1763-1771.
- WULLNER, U., TESTA, C. M., CATANIA, M. V., YOUNG, A. B., & PENNEY, J. B., JR. (1994). Glutamate receptors in striatum and substantia nigra: effects of medial forebrain bundle lesions. *Brain Res.* 645(1-2), 98-102.
- YANG, Y., GEHRKE, S., HAQUE, M. E., IMAI, Y., KOSEK, J., YANG, L., BEAL, M. F., NISHIMURA, I., WAKAMATSU, K., ITO, S., TAKAHASHI, R., & LU, B. (2005). Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. *Proc.Natl.Acad.Sci.U.S.A* 102(38), 13670-13675.
- YAO, H. H., DING, J. H., ZHOU, F., WANG, F., HU, L. F., SUN, T., & HU, G. (2005). Enhancement of glutamate uptake mediates the neuroprotection exerted by activating group II or III metabotropic glutamate receptors on astrocytes. *J.Neurochem.* 92(4), 948-961.
- YUNG, K. K., SMITH, A. D., LEVEY, A. I., & BOLAM, J. P. (1996). Synaptic connections between spiny neurons of the direct and indirect pathways in the neostriatum of the rat: evidence from dopamine receptor and neuropeptide immunostaining. *Eur.J.Neurosci.* 8(5), 861-869.

ZARRANZ, J. J., ALEGRE, J., GOMEZ-ESTEBAN, J. C., LEZCANO, E., ROS, R., AMPUERO, I., VIDAL, L., HOENICKA, J., RODRIGUEZ, O., ATARES, B., LLORENS, V., GOMEZ, TORTOSA E., DEL, SER T., MUNOZ, D. G., & DE YEBENES, J. G. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann.Neurol.* 55(2), 164-173.

ZETTERSTROM, T., SHARP, T., COLLIN, A. K., & UNGERSTEDT, U. (1988). In vivo measurement of extracellular dopamine and DOPAC in rat striatum after various dopamine-releasing drugs; implications for the origin of extracellular DOPAC. *Eur.J.Pharmacol.* 148(3), 327-334.

ZHANG, H. & SULZER, D. (2003). Glutamate spillover in the striatum depresses dopaminergic transmission by activating group I metabotropic glutamate receptors. *J.Neurosci.*19;23(33), 10585-10592.

ZIGMOND, M. J., ABERCROMBIE, E. D., BERGER, T. W., GRACE, A. A., & STRICKER, E. M. (1990). Compensations after lesions of central dopaminergic neurons: some clinical and basic implications. *Trends Neurosci.* 13(7), 290-296.

ZIMPRICH, A., MULLER-MYHSOK, B., FARRER, M., LEITNER, P., SHARMA, M., HULIHAN, M., LOCKHART, P., STRONGOSKY, A., KACHERGUS, J., CALNE, D. B., STOESSL, J., UTTI, R. J., PFEIFFER, R. F., TRENKWALDER, C., HOMANN, N., OTT, E., WENZEL, K., ASMUS, F., HARDY, J., WSZOLEK, Z., & GASSER, T. (2004). The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval. *Am.J.Hum.Genet.* 74(1), 11-19.

ZIV, I., ZILKHA-FALB, R., OFFEN, D., SHIRVAN, A., BARZILAI, A., & MELAMED, E. (1997). Levodopa induces apoptosis in cultured neuronal cells--a possible accelerator of nigrostriatal degeneration in Parkinson's disease? *Mov Disord.* 12(1), 17-23.

ZUCH, C. L., NORDSTROEM, V. K., BRIEDRICK, L. A., HOERNIG, G. R., GRANHOLM, A. C., & BICKFORD, P. C. (2000). Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. *J.Comp Neurol.* 427(3), 440-454.

## Appendix I

### Materials

#### Standard solutions

Phosphate-buffered saline (PBS)	137 mM NaCl, 2.7mM KCl, 1.8mM KH <sub>2</sub> PO <sub>4</sub> , 10mM Na <sub>2</sub> HPO <sub>4</sub> , pH 7.6
Tris-buffered saline (TBS)	50 mM Tris, 150 mM NaCl, pH 7.6
Blocking buffer	1% BSA in 0.5M TBS and 10% sodium azide, pH 7.6

#### Reagents and consumables

<u>Material</u>	<u>Supplier</u>
ABC	Vector
Acetic acid, glacial	Sigma
N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082)	Tocris Cookson
Ascorbic acid	Sigma
D-amphetamine	Sigma
Calcium chloride (CaCl <sub>2</sub> )	Sigma
Cannulae tubing	Coopers
(RS)-alpha-cyclopropyl-4-phosphonophenylglycine (CPPG)	Tocris Cookson
Cresyl violet	Sigma
(S)-3,4-dicarboxyphenylglycine ((S)-3,4-DCPG)	Tocris Cookson



Dental cement - De Trey 'Rapid Repair'	Skillbond Direct
Desipramine	Sigma
Diaminobenzidine tetrahydrochloride (DAB)	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
DPX mounting medium	Sigma
Ethanol, absolute (HPLC grade)	Sigma
Glacial acetic acid	Sigma
Histoclear	Sigma
Hydrochloric acid (HCl)	Sigma
Hydrogen Peroxide (30%)	Sigma
6-hydroxydopamine (6-OHDA)	Sigma
Isoflurane	Abbott animal health
L(+)-2-amino-4-phosphonobutyric acid (L-AP4)	Tocris Cookson
Magnesium Chloride (MgCl <sub>2</sub> )	Sigma
Methanol, absolute (HPLC grade)	Fisher
Microscope slides – Superfrost plus	VWR
Microscrews, 1.3mm	Clerkenwell
Microsyringe, 5 µl Hamilton 7000 series	VWR
Microsyringe, 10µl Hamilton 700 series	VWR
Microtubes, 1.5ml Safe-Lock	VWR
Normal goat serum	Vector
Paraformaldehyde (PFA)	Sigma
Pargyline	Sigma
Phosphate buffered saline tablets	Sigma
Pipette tips	G.E. Healthcare

Portex tubing	Portex
Potassium chloride (KCl)	Sigma
Potassium hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	Sigma
Sodium azide	Sigma
Sodium chloride (NaCl)	Sigma
Sodium Hydroxide (NaOH) crystals	Sigma
Sodium phosphate, monobasic ( $\text{NaH}_2\text{PO}_4$ )	Sigma
Sodium phosphate, dibasic ( $\text{NaH}_2\text{PO}_4$ )	Sigma
Tris base / Trizma	Sigma
Tryptan blue dye	Sigma
( $\pm$ )-cis-2-(3,5,-dichlorophenylcarbamoyl) cyclo-hexanecarboxylic acid (VU0155041)	Ascent Scientific

## Supplier Contacts

Abbot animal health	Abbott Laboratories Ltd, Kent, UK
Abcam	Abcam, Cambridge, UK
Amersham	Amersham Pharmacia Biotech UK Ltd, Bucks, UK
Ascent Scientific	Ascent Scientific Ltd (UK), Bristol, UK
B & K	B & K Universal Limited, Hull, UK
Chemicon/ Millipore	Millipore (U.K.) Ltd, Watford, UK
Clerkenwell Screws	Clerkenwell Screws, Farringdon, London, UK
Coopers	Coopers Needleworks, Birmingham, UK
Dako	Dako UK Ltd, Cambs, UK
Fisher	Fisher Scientific UK Ltd, Leics, UK
GE Healthcare	Chalfont St Giles, Bucks, UK
GraphPad	GraphPad software, San Diego, CA, USA
KD Scientific	Distributed by Presearch, Hamps, UK
Kopf	Tujung, CA
Leica	Leica Microsystems (UK) Ltd, Milton Keynes, UK
Portex	Distributed by Scientific Laboratory Supplies (SLS), Nottingham, UK
Scion Corporation	Scion Corportation, Maryland, USA
Sigma	Sigma Chemical Company Ltd, Poole, Dorset, UK
Skillbond	Skillbond direct, Bucks, UK
RS Components	RS Components Ltd, Northants, UK
Tocris Cookson	Tocris Cookson Ltd, Avonmouth, Bristol, UK
US Biological	Distributed by Europa Bioproducts Ltd, Ely, Cambs, UK

Vector	Vector Laboratories Ltd, Peterborough, UK
VWR	VWR International Ltd, Leics, UK
Zeiss	Carl Zeiss Ltd, Welwyn Garden City, Herts, UK